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ANALYSIS OF THE ELECTRICAL ACTIVITY OF THE OLFACTORY EPITHELIUM

By

DAVID OTTOSON

STOCKHOLM 1956

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"If we possessed a true, well established and undoubted theory concerning the function of the nerves in the human body, the principles of smell would be much easier to understand. So far, we do not know for certain whether the action of nerves takes place by means of a subtle fluid, which is instantaneously conveyed from the beginning of the nerves to their ends, or whether, as some think, some sort of vibrating movements is produced in them, or whether the stimulation should be considered as the only sufficient cause, to say nothing of the new opinion concerning the electrical force."[1]

[1] *Carl von Linné,* Odores Medicamentorum. Dissertatio. Stockholm 1752. Translation from the Swedish edition published by Svenska Linné-Sällskapet (1954). Linné's treatise, in which the earliest classification of odorous substances was presented, is often erroneously referred to as having been published in 1756. The original paper which was written in Latin appeared in 1752 but was reprinted four years later in Amoenitates Academicae, Ed. I, Vol. 3, Stockholm and Amsterdam-Leyden.

Introduction

Electrophysiological studies on the sense of smell have been greatly hampered by the inaccessibility of the olfactory end organs and by the fact that the afferent nerve fibres are very short and extremely thin. Investigations on the function of the olfactory system have therefore been mainly restricted to studies of the discharge in the olfactory bulb.

The electrical activity of the mammalian olfactory bulb has been analysed by ADRIAN (1942, 1950, 1951), who showed that strong olfactory stimulation evoked a series of regular oscillations which suppressed the spontaneous activity in the bulb. These oscillations - the induced waves - were obtained from the outer layers of the bulb and were assumed to represent the activity in the dendritic network of the bulb. From the deeper layers of the bulb bursts of impulses were recorded when the animal inspired odorized air. These spike potentials were ascribed to the activity of the mitral cells and their axons. ADRIAN (1951, 1953) observed that different parts of the bulb were activated when different odorous substances were used; in low concentrations water soluble substances elicited responses mainly in the anterior part of the bulb, while substances soluble in lipoids evoked discharges in the posterior part. The spatial distribution of the responses was assumed to be due to a localized projection of different areas of the olfactory epithelium on to the bulb. This hypothesis has been confirmed in histological studies by LE GROS CLARK (1951). Apart from the differences in the spatial distribution of the responses ADRIAN (1951) found that there were differences in latency as well. In studies on the discharge of the mitral cells it was further found that there were units exhibiting a specific sensitivity to particular substances. However, the investigations brought no evidence that there exists a small number of basic odours out of which all others can be composed.

On the basis of the results obtained in the investigations on the electrical activity in the olfactory bulb in different animals,

ADRIAN (1948, 1951, 1952, 1953, 1954) advanced a theory according to which the discrimination of odours depends on differences in the temporal and spatial pattern of excitation as well as on a differential sensitivity of various types of receptors.

The observations made by ADRIAN on the time relations and spatial distribution of the responses in the olfactory bulb have later on been confirmed by MOZELL and PFAFFMANN (1954). The activity of single units in the bulb has been studied by WALSH (1955), who reported that only a small number of the active cells responds to olfactory stimulation, the activity of the others being either spontaneous or related to the respiratory cycle.

Studies of the discharge in higher olfactory centres have contributed to the knowledge of the fibre connections of the olfactory system but have yielded little information on the function of the peripheral olfactory sensory apparatus.

In the course of some experiments in which the discharge in the bulb of the rabbit was studied it was observed (OTTOSON, 1954) that olfactory stimulation evoked a slow sustained positive potential apart from the potential changes described by earlier authors. This response, which was obtained also when the animal breathed room air, increased in amplitude up to a definite maximum when the odour intensity of the air was increased and showed little sign of adaptation. The induced waves described by ADRIAN (1942, 1950) appeared superimposed upon this slow potential. In the course of the studies on the characteristics of the response observations were made which led to the assumption that the origin of the potential was located in the sensory epithelium of the nasal mucosa. This view was supported by the observation that a slow sustained potential of the same shape and time course as that recorded from the bulb was obtained when recording was made from the olfactory nerve or directly from the nasal mucosa after removal of the bulb.

Owing to the complex structure of the olfactory perceptive apparatus in the rabbit this animal was not very well suited for the analysis of the slow potential in the mucosa and another type of preparation was therefore looked for, in which the receptor sheet could be reached more readily. The frog offered such a

preparation. In this animal the olfactory epithelium can easily be exposed, and furthermore recordings can be made simultaneously from the nasal mucosa and from the olfactory bulb.

The aim of the present work has been to analyse the electrical activity of the olfactory epithelium. In the first part of this paper an account will be given of the results obtained in studies on the origin of the slow potential. The characteristics of the response, its shape, time course and changes in latency and in amplitude with changes of stimulus strength will be described in the second part. In the third part a series of experiments will be reported which were carried out in order to analyse certain functional properties of the olfactory epithelium: the adaptation to continuous and repetitive stimulation, the recovery and further the selectivity of fatigue caused by different odorous stimuli. Finally some experiments will be described in which the mechanisms of olfactory stimulation were studied.

Data on the frog's sense of smell

Since the frog possesses a well developed olfactory epithelium it might be expected that this animal can recognize odours. Studies on the behaviour of the frog have, however, given no definite answer to the question whether it can smell or not. There is no evidence that the olfactory organ is of any decisive importance for the animal in seeking food (SCHAEFFER, 1911; RISSER, 1914). In order to attract the frog, the food must be in motion, but it does not seem to be chosen or rejected because of its odours. Nor did RISSER (1914) find any evidence that the toad in taking food is guided by olfactory stimuli, but he was able to demonstrate that definite motor reactions were elicited in this animal by olfactory stimulation. In investigations on the behaviour of other amphibians it has been conclusively shown that they make use of the sense of smell in seeking food. Thus it was found by KRAMER (1933) that *Xenopus* is attracted by the odour of food and MATTHES (1927) demonstrated that *Triton* is able to trace odorous food both when living on land and in water.

From the anatomical point of view the frog has been classified as microsmatic (GAUPP, 1899), *i.e.* belonging to the class of animals which in comparison with macrosmatic animals, for instance the dog, has a less well developed sense of smell. This classification is based mainly on differences in the structure of the central olfactory system in different species. The peripheral sensory apparatus - the olfactory epithelium - in the frog is identical in structure with that in higher vertebrates.

The anatomy of the frog's olfactory organ

The nasal cavities. When compared with the structure of the olfactory organ in higher vertebrates the nasal cavities of the frog are fairly simple. *Apertura nasalis externa* (see Fig. 4) opens via *vestibulum nasi* into a large cavity, *cavum principale nasi,* the walls of which are lined with sensory epithelium. By *septum nasi* this space is separated from the nasal cavities of the other side. From the ventral wall, *i.e.* the bottom of the main cavity, there rises a rounded ridge, *eminentia olfactoria,* at the lateral border of which is found the opening *(apertura nasalis interna)* into the buccal cavity. In the lateral direction the main cavity narrows into *recessus lateralis,* the walls of which are covered with respiratory epithelium. The lateral recess extends in oral and medial directions into a tubular space, *recessus medialis,* the epithelium of which is of the same type as that in the main cavity. The regions lined with sensory epithelium have a slightly yellow colour and are therefore easily distinguished from those where the epithelium is of respiratory type, these latter regions being pink.

The sensory epithelium. The olfactory epithelium consists of three types of cells: the sensory cells or the primary olfactory neurons, the supporting cells and the basal cells (see Fig. 1). The sensory cells are bipolar neurons, the nuclei of which are located at different heights in the epithelium. From the peripheral part of the cell body there extends a thin cylindrical process terminating at the external limiting membrane, where it forms a vesicular

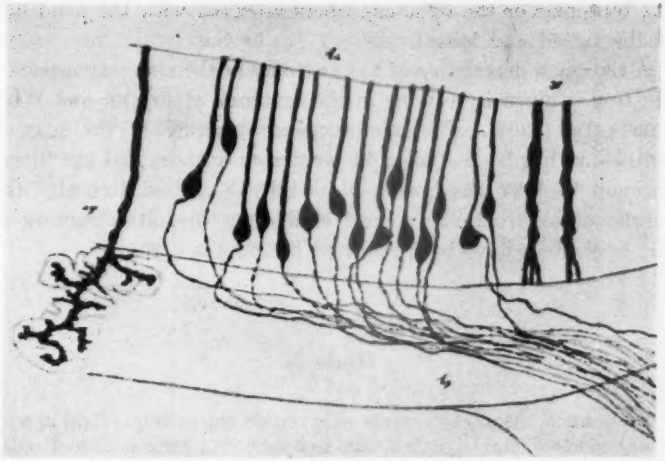

Fig. 1. Olfactory epithelium showing the fusiform cell bodies of the primary olfactory neurons. The peripheral cylindrical processes extend to the outer limiting membrane. The olfactory hairs are not seen. The thin nerve fibres arise from the proximal poles of the cells and pierce the basal membrane. To the left a Bowman's gland. To the right two sustentacular cells. Mouse. GOLGI'S method. After RETZIUS 1892.

enlargement, *vesicula olfactoria*. From this structure 6—12 hair-like extremely thin filaments, the olfactory hairs, protrude into the mucus covering the epithelium. According to HOPKINS (1926) who studied the structure of the living olfactory epithelium in frog there are two types of olfactory hairs. One kind is 20—50 μ in length and does not usually reach the surface of the mucous layer. They show a slow waving motion which differs from that of cilia. The hairs of the other type are non-motile 75—200 μ long filaments which to a great extent of their length lie at the surface of the epithelium. The mucus is secreted by numerous glands in the epithelium and protects the olfactory hairs from drying. The olfactory nerve fibre, the diameter of which is less than 1 μ, extends from the proximal part of the cell body. Bundles of fibres, *fila olfactoria*, form the olfactory nerve which passes through *canalis olfactorius* into the cranial cavity. Here the olfactory nerve enters into the olfactory bulb. Within the bulb

the terminals of the olfactory fibres interlace with the dendrites of the tufted and mitral cells.

A thorough description of the anatomy of the olfactory organ of the frog is given by GAUPP in the textbook of ECKER and WIEDERSHEIM (1904). The microscopical structure of the sensory epithelium has been studied by numerous authors and the literature up to 1927 has been reviewed by KOLMER. Recently the results of electronmicroscopical studies on the ultrastructure of the epithelium have been reported by BLOOM (1954).

Methods

Preparation. Most experiments were carried out on frogs *(Rana temporaria)* collected during the autumn and kept in a tank in a cool cellar. After decapitation of the frog the nasal mucosa was exposed by removal of the dorsal wall of the main cavity of the olfactory organ. The preparation was kept in a moist chamber of cotton wool irrigated with Ringer's solution.

Stimulation. The experimental arrangement for stimulation is shown in Fig. 2. The expelled air from a respiration pump was used for the stimulation of the olfactory epithelium. At each stroke of the pump about 50 cc of air was forced into the air line. The greater part of the air was blown out through an outlet (b) while the rest was filtered through activated carbon (c and i) before it passed into a 150 cc test bottle (k) containing 10—15 cc of an aqueous solution of the odorous substance. The odorous air was blown on to the nasal mucosa through a glass pipette (r_1), which was connected to the outlet of the test bottle. The rubber tubes connecting the charcoal filter with the test bottle and the pipette with the test bottle were made as short as possible and boiled in water with activated carbon in order to reduce their odour. The tip of the pipette was usually kept at a distance of 5—8 mm from the surface of the epithelium. The volume of a single air puff with which the epithelium was stimulated was about 1 cc. By introducing glass capillaries (f) of different sizes in the air line, the volume of air expelled through the pipette at each stroke of the pump could be varied from 0.1 to 1 cc.

In most experiments butanol (n-butanol) was used as odorous substance owing to its comparatively high solubility in water. For practical reasons a limited number of solutions of different concentrations had to be used; 0.001 M, 0.005 M, 0.01 M, 0.025 M, 0.05 M, 0.1 M, 0.25 M were chosen. About 15 cc of the solutions were kept in test bottles that had been

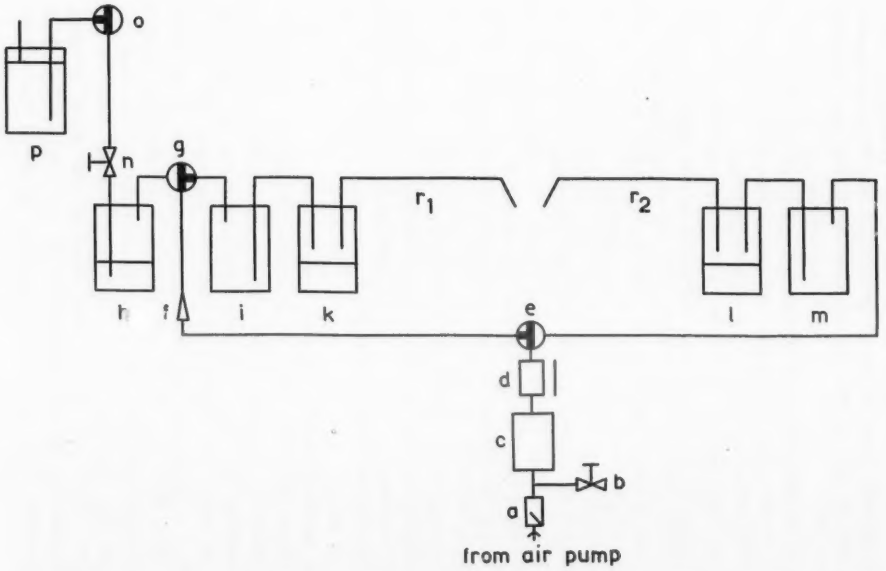

Fig. 2. Diagram of experimental arrangement used for stimulation. a, valve; b, outlet; c, charcoal filter; d, pressure recording chamber; e, stopcock; f, glass capillary; g, stopcock; h, water bottle; i, charcoal filter; k, test bottle; l, test bottle; m, charcoal filter; n, valve; o, stopcock; p, water tank; r_1 and r_2 outblow pipettes.

carefully cleaned, and when the stimulus strength had to be altered the test bottle (k) was replaced by another bottle containing a solution of a different concentration. When the effect of different stimulus intensities was studied the stimulations were applied in the order from lower to higher stimulus strengths. At the end of such a series of stimulations the pipette (r_1) was replaced by a clean one. The solutions in the test bottles as well as the charcoal in the filters were regularly renewed.

The duration of each separate outblow of air through the pipette was about 0.9 sec. After each stimulation the preparation was usually left unstimulated for 3—5 min in order to avoid adaptation.

No attempts were made to determine the absolute stimulus strength, *i.e.* the concentration of the odorous substance in the stimulating air. Since this concentration is proportional to the concentration of the substance in the solution, the data on stimulus strengths are given in units corresponding to the molarity of the solutions.

When substances were used (*e.g.* oil of cloves) that could not be dissolved in water to such an extent as to give solutions of the desired odour intensities paraffin oil was used as solvent. In some experiments small pieces of filter paper were soaked in the substance and then placed in the test bottle. By placing a varying number of such pieces in the bottle the odour intensity of the gas in the bottle could be adjusted. For obvious reasons it was impossible by this crude method to alter the stimulus strength in a well defined way. No attempts have been made in the present investigation to study the relation between the magnitude of the response and the stimulus strength for substances which could not be dissolved in water.

The purest available substances were used.

The continuous stimulation used in the studies of olfactory adaptation was brought about by passing air through the test bottle at a constant rate. This was done by letting water run from a tank (p) into the bottle (h), the air of which was expelled through the charcoal filter (i). By altering the flow of water the velocity of the stimulating air stream could be changed. This flow was usually adjusted to 1 cc/sec. The duration of the stimulation was comparatively long (3—30 sec) in these experiments, and the large amount of filtered air passed into the test bottle therefore caused a gradual diminishing of the odour intensity of the air expelled during each stimulation. In order to avoid such an effect 500 cc bottles were used containing about 50 cc of the odorous solution.

A second stimulus set-up consisting of a charcoal filter (m) and a test bottle (l) connected to the pipette (r_2) was used in the experiments in which two types of odorous substances were alternatively used for stimulation (see section D, Part III). The substances were kept in the test bottles (k and l), and by turning the stopcock (e) in the interval

between two strokes of the air pump a rapid change of the stimulus was achieved.

Electrodes. Agar—AgCl—Ag electrodes were used in most experiments, one electrode (tip diameter 0.1—0.2 mm) being placed at the surface of the olfactory epithelium and the other in contact with the moistened cotton wool on which the preparation was placed. The preparation was usually not earthed. The electrodes were connected to the input of a symmetrical direct coupled amplifier (HAAPANEN, 1953), and the responses photographed from a double-beam cathode-ray tube on slowly moving film. In a few experiments the stimulation was marked on the lower beam of the oscillograph (see *e.g.* Fig. 5) by recording the small increase of pressure occurring in the system at each stroke of the pump. This was performed by recording the changes in capacitance between two metallic plates, one of which was fixed on the thin rubber membrane of the chamber (d). It should be mentioned, however, that exact measurements of the latency of the response could not be made in this way, since the increase of pressure in the system started before the stimulating air stream reached the nasal mucosa. The method used for such measurements will be described in section G, Part II.

In some experiments capillary microelectrodes were used filled with 3 M-KCl (tip diameter about 0.5 μ). In these experiments the electrodes were connected to the amplifier via a cathode follower (HAAPANEN and OTTOSON, 1954) and the preparation earthed.

In all recordings an upward deflection indicates a negativity of the different (active) electrode.

PART I

The Response of the Olfactory Epithelium
General Description and Origin

A. *General description of the response to olfactory stimulation*

A slow negative purely monophasic response was recorded from the olfactory epithelium when a small amount of odorized air was blown into the nasal cavities. As illustrated in Fig. 3 this potential was characterized by a steep rising phase followed by a slower decline towards base-line. The amplitude of the response was graded to the odour intensity of the stimulating air and amounted to 2—3 mV at medium stimulus strength. The polarity and general shape of the potential was independent of the type of the odorous stimulus, although certain differences in shape were noticed between responses evoked by different odorous substances. A detailed description of these differences is given in section F in Part II.

The potential was obtained only from the yellow regions of the nasal mucosa, whereas no response or a very small one was recorded from areas in which the mucosa was pink, *i.e.* in the lateral recess and in *cavum medium*.

If the preparation was carefully protected from drying and not subjected to frequent stimulation or stimulation at high intensity it gave reproducible responses for 6—8 hours and remained excitable for more than 12 hours.

A few experiments were also carried out on toads in order to study whether a similar potential was generated by the olfactory epithelium in this animal. It was found that stimulation with odorized air evoked a response identical in shape and time course with that obtained in the frog. The responses recorded from the toad's nasal mucosa were, however, generally of smaller amplitudes than those obtained from the frog.

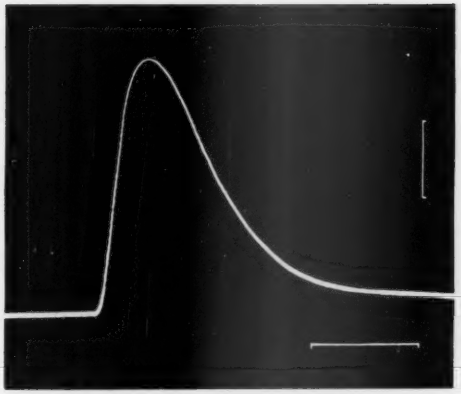

Fig. 3. Response of olfactory epithelium to stimulation with odorized air. Stimulus: butanol. Volume of stimulating air 0.5 cc. Vertical line 1 mV. Time bar 2 sec. Upward deflection in this and the following records indicates negativity of the active electrode.

B. *Stimulation of restricted areas of the nasal mucosa*

In the course of the first series of experiments it was noticed that the responses obtained from different regions of the olfactory epithelium in the main cavity differed considerably in amplitude although equal stimulus intensities were used. In order to study these differences more closely attempts were made to restrict the stimulation to a small area of the epithelium. For this purpose an outblow pipette with a small tip (diameter 0.1 mm) was used, the pipette being placed so that the distance between the tip and the surface of the epithelium was 1—2 mm. Recordings were made with a microelectrode at the point of the epithelium towards which the stimulating air stream was directed. The response obtained in this way apparently represented the activity of a rather small area of the epithelium, since no significant response was obtained when the air stream was directed towards a point 2—3 mm apart from that at which the electrode was placed.

Fig. 4 shows a schematic diagram of the frog's nasal cavities, the dotted area indicating regions where the olfactory mucosa

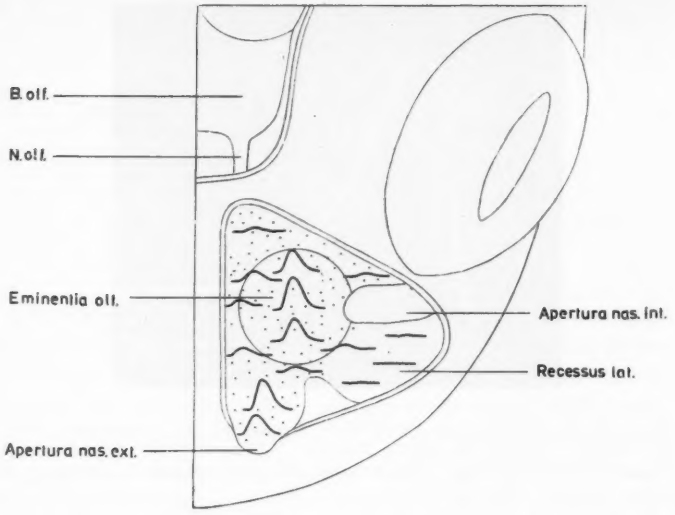

Fig. 4. Schematic diagram of the preparation showing responses to stimulation of restricted areas of the mucosa. Dotted regions indicate sensory epithelium.

is of yellow colour. The superimposed records in this diagram illustrate the responses obtained when different areas of the nasal mucosa were stimulated. As is seen responses were achieved only from the yellow regions representing areas with sensory epithelium. The boundaries between the parts within which responses were obtained and those which gave no responses coincided well with the boundaries between the regions of different colours. The greatest potentials were recorded from the top of *eminentia olfactoria* and from the epithelium in the anterior part of the main cavity close to *vestibulum nasi*, whereas the responses obtained from the regions surrounding *eminentia olfactoria* were of considerably smaller amplitude. From the epithelium of the excised dorsal wall of the main cavity responses of medium amplitude were recorded.

C. *The effect of cocaine*

From investigations on the activity of the compound eye of the water beetle (BERNHARD, 1942) as well as on the discharge recorded from the isolated muscle spindle (KATZ, 1950) or from the Pacinian corpuscle (GRAY and SATO, 1953), it is established that sensory end organs are not susceptible to the action of local anaesthetics; at a concentration of the drug sufficiently high to cause a block of the impulse conduction in the nerve fibres the receptors still remain excitable. The dendritic terminals of the stretch organ in the lobster exhibit similar properties as revealed by the persistence of the generator potential after the impulse transmission is blocked by novocaine (EYZAGUIRRE and KUFFLER, 1955 a). By use of local anaesthetics the activity of sensory end organs can thus be studied without the interference of the discharge in the nerve fibres.

In view of these observations it appeared to be of interest to examine the effect of cocaine on the slow potential recorded from the olfactory epithelium. Apart from the ordinary recording from the nasal mucosa, the activity of the olfactory bulb was also led off in these experiments. As will be described thoroughly in another paper a slow potential with superimposed oscillations is obtained from the olfactory bulb when the nasal epithelium is stimulated with odorized air. As this response can be evoked only when the conduction in the olfactory nerves is intact it can be regarded as an index of the transmission of impulses from the epithelium to the bulb. The discharge in the olfactory nerve fibres is not easy to record due to their small diameters, whereas there are no difficulties in recording the response in the bulb.

Butanol was used as test stimulus and the effect of a 0.5 % cocaine-Ringer's solution was studied by recording in one series of experiments the response of the olfactory epithelium and - in another series of experiments - the response of the olfactory bulb.

It was found that the response of the olfactory epithelium was reduced to about 60 per cent of its original amplitude after the nasal mucosa had been treated with the cocaine solution for a few minutes (Fig. 5 A and B). However, this effect was transient

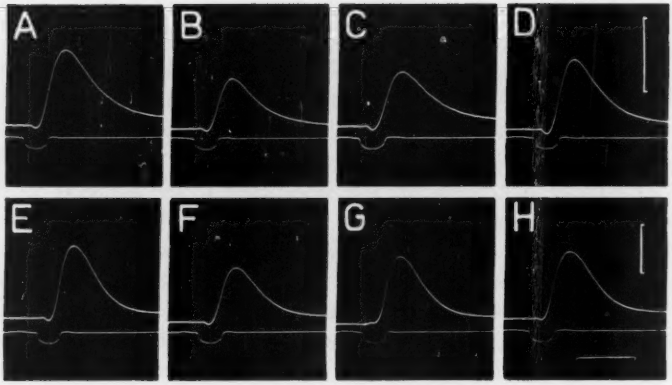

Fig. 5. The effect of cocaine (A—D) and of Ringer's solution (E—H). Records of responses to stimulation with butanol: A, before; B, 3 min; C, 10 min; D, 30 min after application of 0.5 % cocaine solution on the mucosa. E—H from another preparation. E, before; F, 3 min; G, 10 min; H, 30 min after treatment with Ringer's solution. Stimulation marked on lower beam. Stimulus: 0.01 M butanol. Volume of air 1 cc. Vertical line in D and H 2 mV. Time bar 2 sec.

and the response usually regained its original amplitude within 30—45 min (D). The diminution of the potential was not due to a specific action of the drug since a similar effect was obtained by Ringer's solution alone (Fig. 5 E—H, see further discussion page 29).

The response of the olfactory bulb was also abolished after a few minutes' cocainization of the nasal mucosa but did not reappear within the 30—45 min, when the sensory epithelium response was already completely restored. This disappearance of the bulb response must be due to the fact that no nerve impulses were transmitted up to the bulb - apparently a blocking effect of the cocaine.

On the basis of these results and in view of the previously mentioned observations on the insusceptibility of sensory terminals to the action of local anaesthetics, it appears justified to infer that the slow response of the olfactory epithelium originates in the receptors.

D. *The effect of antidromic stimulation of the olfactory nerve*

In the experiments described above the observation was sometimes made that the response did not regain its full original amplitude after the epithelium had been treated with cocaine. It could therefore not be entirely excluded that a small fraction of the potential came from the nerve fibres.

It has already been pointed out that in the frog the olfactory nerve fibres converge into one single nerve, *n. olfactorius*, which is easily accessible within the cranial cavity (see Fig. 4). When this nerve is electrically stimulated, all fibres can therefore be simultaneously excited. By antidromic stimulation of the olfactory nerve it would thus be possible to analyse whether any fraction of the slow response evoked by olfactory stimulation of the epithelium originates in the nerve fibres.

When the same amplification was used as for the recording of the slow response of the olfactory epithelium no potential changes - except those due to the shock artefacts - could be recorded from the nasal mucosa on electrical stimulation of the olfactory nerve. Nor could any significant change of the response to stimulation with odorized air be observed when the nerve at the same time was electrically stimulated at different frequencies and strengths.

It was therefore concluded that no significant fraction of the response set up in the sensory epithelium by olfactory stimulation originates from the nerve fibres. The results further indicate that the structures in which the potential arises cannot be thrown into action by antidromically conducted nerve impulses.

E. *Recordings from different depths in the olfactory epithelium*

In the literature on olfaction different opinions are to be found as to which part of the primary olfactory neuron constitutes the receptor proper. Most authors do not clearly define which struc-

ture is meant when discussing the sensory element, and the reader is left to guess.

It is generally agreed that the olfactory hairs play an important rôle in the processes of perception of odours. The hairs are, however, usually lost in sections of fixed preparations of the olfactory epithelium and have therefore often been disregarded in discussions on the mechanisms of olfactory stimulation. In descriptions on the structure of the olfactory epithelium the entire primary neuron within the epithelium is usually called the olfactory receptor. Since the cell body is presumed to serve reception as well as conduction the olfactory end organs are considered to differ fundamentally from other types of vertebrate receptors. According to this opinion the olfactory receptors would represent a primitive type of sensory cells, such as those found in lower animals. By some authors the peripheral extension has been defined as the true receptor and since the length of this part of the cell differs greatly from one neuron to another two types of receptor cells have been distinguished, olfactory rods and cones (see *e.g.* DOGIEL, 1886).

In a series of experiments recordings were made with a microelectrode from different depths within the epithelium in order to explore in which part of the primary neuron the response originated. The microelectrode was inserted stepwise (25 μ) into the mucosa and the response recorded at each level. The recordings were usually made from the epithelium on the top of *eminentia olfactoria* and care was taken to insert the electrode perpendicularly to the surface of the mucosa. The distance between the surface of the mucous layer and the depth to which the tip of the electrode was advanced was measured with a micrometer gauge mounted on the micromanipulator.

The potential obtained at a depth of 25 μ was found to be slightly lower than that recorded from the surface of the epithelium. As the electrode was advanced further the amplitude of the potential continuously declined the decrease being more rapid after the 50 μ level was passed. When recordings were made at a depth of 150—175 μ responses were obtained which amounted only to 15—20 per cent of those recorded at the surface. The records in Fig. 6 illustrate the responses obtained in one of these ex-

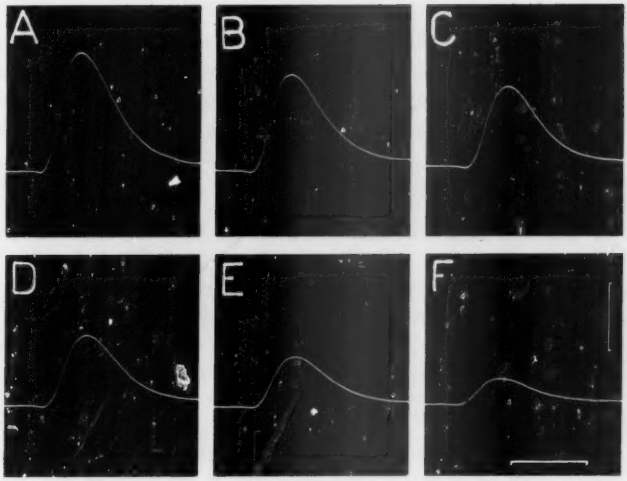

Fig. 6. Records of potentials recorded from different depths in the olfactory epithelium. A, response obtained at the surface of the mucous layer; B, at a depth of 50 μ; C, 75 μ; D, 100 μ; E, 125 μ; F, 150 μ. Stimulus: 0.01 M butanol. Volume of air 1 cc. Vertical line in F 2 mV. Time bar 2 sec.

periments. The results differed but slightly from one experiment to another, if the insertions were made within a restricted area of the epithelium. The curve in Fig. 7 represents the results achieved in 10 experiments in which the insertions were made at the top of *eminentia olfactoria*. The curve is obtained by plotting the amplitude of the potential as a percentage of the response recorded at the surface against the distance from the depth, at which the recording was made, to the surface of the mucous layer. As shown the amplitude of the potential decreases continuously the further from the surface the response is led off, although the decrement is less marked for the first 50 μ.

According to v. BRUNN (1875) the thickness of the olfactory epithelium of the frog is 140 μ when measured from the outer limiting membrane to the basal membrane. HOPKINS (1926) found that the mucous layer covering the living epithelium in the frog is usually 25 μ thick with variations from 10 to 60 μ. In

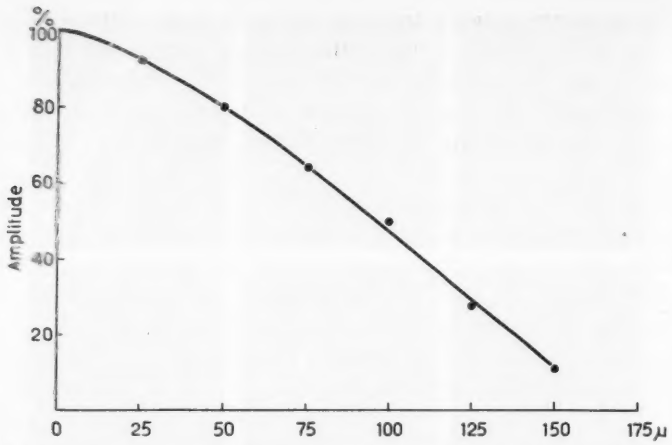

Fig. 7. Diagram showing the relation between the amplitude of the potential and the distance from the surface of the mucous layer to the recording point in the epithelium. Ordinate: amplitude in per cent of potential recorded at the surface of the epithelium. Abscissae: distance in micra from the recording point to the surface of the mucous layer.

the present investigation no measurements were made of the thickness of the mucosa in the preparations used, but according to the observations of v. BRUNN and HOPKINS the distance between the surface of the mucous layer and the basal membrane in the olfactory epithelium is 150—200 μ.

Although the measurements with the micrometer did not give exact information as to the depth from which the recordings were made, certain assumptions may nevertheless be made on the basis of the results achieved. If we assume that the thickness of the mucous layer is about 25 μ, the amplitude of the potential apparently changes only slightly until the electrode has passed through this layer and reached a depth corresponding to the position of the outer limiting membrane. The amplitude of the response rapidly declines at a further insertion of the electrode, *i.e.* when the tip of the electrode passes along the peripheral extensions of the neurons, penetrates the layer of cell bodies and approaches the basal membrane.

The results indicate that the response originates from structures located close to the surface of the epithelium. If these structures - as it appears - are identical with the olfactory hairs, there seems to be little reason to designate the olfactory receptors as representing a primitive type of end organs.

F. *The effect of distilled water, ether and chloroform*

As mentioned, the olfactory epithelium is covered with a layer of mucus in which the olfactory hairs are embedded. According to most authors (see *e.g.* KOLMER, 1927) the hairs do not penetrate through this layer, and thus they are not in direct contact with the air in the nasal cavities. This fact implies that the stimulating substance has to pass through a more or less thick layer of mucus before reaching the receptors. It has therefore been suggested that there is no fundamental difference between the mode of olfactory stimulation in air-breathing and water-inhabiting vertebrates. This matter was a subject for controversy for a long time, and great attention was paid to the question whether any olfactory sensations could be evoked by the instillation of solutions of odorous substances in the nasal cavities (see WEBER, 1847; FRÖHLICH, 1851; ARONSOHN, 1886; NAGEL, 1894; ZWAARDEMAKER, 1895). Later on this problem was taken up again by HOPKINS (1926) in investigations on the structure of the living olfactory epithelium of air-breathing and water-inhabiting animals with special regard to the sensitivity of the nasal mucosa to the action of certain substances. Previously it had been reported by SCHULTZE (1862) that the olfactory hairs were rapidly destroyed by water, whereas no effect could be noticed upon the cilia of the respiratory epithelium. HOPKINS found that distilled water caused a destruction which was, however, confined to the longer olfactory hairs. When, on the other hand, ether or chloroform vapour was blown into the nasal cavities of the frog both types of olfactory hairs were severely damaged.

These observations indicate that a selective destruction of the hairs might be obtained by treating the nasal mucosa with dis-

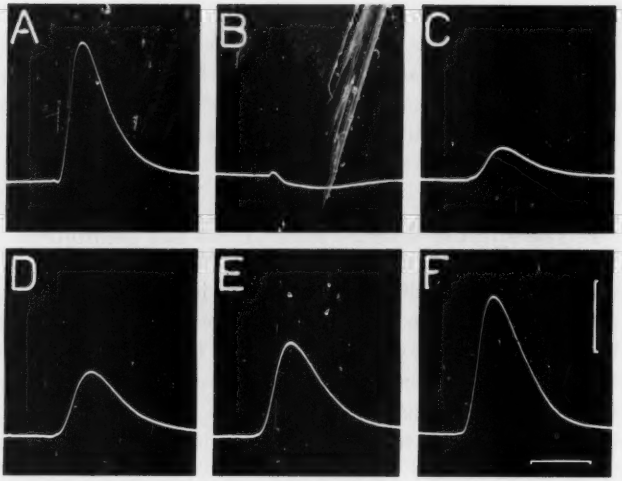

Fig. 8. The effect of ether. Records of responses to stimulation with butanol: A, before; B, immediately after ether vapour was blown into the nasal cavity; C, 10 min; D, 20 min; E, 30 min; F, 60 min later. Volume of air 1 cc. Vertical line in F 1 mV. Time bar 2 sec.

tilled water, ether or chloroform. In this manner it would thus be possible to examine the functional importance of the hairs.

It was found that a treatment of the nasal mucosa with distilled water for 1—2 min caused a transient block of the response to stimulation with butanol. This effect lasted for 10—15 min, after which a small response could usually be evoked again on stimulation. During the ensuing hour the response became successively greater, but the original amplitude was not regained. If the water was allowed to remain on the mucosa for more than 2 min the response was blocked for a longer period and the recovery was less complete. In many experiments the response never reappeared after the epithelium was treated with water.

If a few cc of ether or chloroform vapour was blown on to the nasal mucosa, no response was evoked by the subsequent stimulation with butanol. The response was, however, blocked only for a few minutes and was usually re-established to its original

amplitude within less than one hour. The result obtained in an experiment with ether is illustrated in Fig. 8. Record A shows the response to butanol. A small amount of ether vapour - approximately 2 cc - was then blown into the nasal cavities. On the subsequent stimulation with butanol no response was elicited (B). The recovery of the epithelium was then tested with stimulations applied at intervals of 5 min. After 10 min a response was obtained (C), the amplitude of which amounted to nearly 25 per cent of the original amplitude of the response, this being gradually restored in the course of one hour, as illustrated by the records D, E and F. If greater amounts of ether vapour were blown on to the nasal mucosa, the blocking effect was of longer duration and the recovery of the epithelium slower. The original amplitude of the response was then usually not regained. The response was irreversibly abolished if the epithelium was exposed to the action of ether vapour for some minutes.

It is possible that the irreversible abolition of the response was caused by a selective destruction of the olfactory hairs, although it cannot be excluded that other parts of the olfactory neurons were also damaged. Anyhow, the remarkable sensitivity of the epithelium to small amounts of ether or chloroform vapour indicates that the blocking effect is produced by the action of these substances on structures located close to the surface of the mucous layer. Although the results give no definite answer to the question as to which part of the neuron constitutes the true receptor, they strongly support the view that the olfactory hairs play an important rôle in the generation of the response of the epithelium.

G. *Discussion*

The slight interest paid to studies of the function of the olfactory end organs is illustrated by the fact that apart from the previously mentioned paper (OTTOSON, 1954) only one report has been published on investigations in which recordings have been made directly from the receptor layer in the nasal mucosa. In a brief communication ADRIAN (1955) recently reported that oscillatory potentials were obtained from the olfactory epithelium

in the rabbit. The oscillations had a frequency of 30—60/sec and were also obtained in recordings across the mucosa. From this observation ADRIAN concluded that the oscillations originated in the olfactory receptors. Under certain experimental conditions similar oscillatory potentials were recorded from the olfactory epithelium in the frog, as will be described in section E in Part III.

Although the results of the previously reported experiments on the rabbit (OTTOSON, 1954) led to the conclusion that the slow response was elicited by stimulation of the olfactory receptors, other possibilities could not be ruled out. Thus, it is well known that there is a potential difference across the mucous membranes in the oral cavity and in the gastrointestinal tract (see *e.g.* BIEDERMANN, 1893). A similar potential difference also exists between the outer layer of the skin and the subepithelial connective tissue layer (see *e.g.* SCHAEFER, 1940; concerning the location of the potential see OTTOSON *et al.*, 1953). From numerous investigations it is further known that the steady potential of mucous membranes (see *e.g.* HERMANN and LUCHSINGER, 1878; BIEDERMANN, 1893) as well as that of the skin (see *e.g.* ORBELI, 1910; UHLENBRUCK, 1924) exhibits slow variations on direct or indirect stimulation. It may be presumed that a similar potential difference exists across the nasal mucosa, and in view of the observations mentioned above it cannot *a priori* be excluded that the slow response obtained on stimulation of the olfactory epithelium is produced - at least partly - by a variation in such a potential. In the rabbit it was difficult to settle this question, since it could not be ascertained exactly from which region of the mucosa the response originated. In the frog, however, the distribution of the potential could easily be examined by stimulating restricted areas of the nasal mucosa, and in this way it was established that only regions with sensory epithelium gave responses.

It is of interest to note that the differences in amplitude between responses obtained from different regions of the sensory epithelium are related to structural differences between the olfactory neurons in these areas. According to GAUPP (1904) the olfactory epithelium is thickest on the top of *eminentia olfactoria* and in the anterior part of the main cavity, while the epithelium on the slopes of *eminentia olfactoria* is less well developed. In

the grove between *septum* and *eminentia olfactoria* as well as in regions close to the lateral recess or *cavum medium* the sensory epithelium is thin or replaced by indifferent epithelium. As seen in Fig. 4 the regions of the nasal mucosa, from which small responses were obtained, coincide with those in which the sensory epithelium is poorly developed. It is likely that the differences in amplitude illustrated in Fig. 4 depend not only on differences in the number of the sensory cells but also on functional differences between the olfactory receptors in different regions of the mucosa. In this connection it may be pointed out that the thickness of the olfactory epithelium varies considerably in different species and the suggestion has been made (see *e.g.* GEREBTZOFF, 1953) that the olfactory acuity is related to the height of the epithelium.

In the rabbit the slow response was not abolished by cocaine (OTTOSON, 1954), and it was therefore concluded that the potential came from the olfactory receptors. Owing to the complex structure of the olfactory organ in the rabbit it was, however, difficult to ascertain that the region of the mucosa, from which the potential was led off, was actually exposed to the action of the drug. However, in the frog the entire sensory epithelium could readily be reached, and furthermore the effect on the impulse conduction in the nerve fibres could be checked by recording the activity of the olfactory bulb. The transient reduction of the response (see Fig. 5) after treatment of the mucosa with the cocaine solution could apparently not be ascribed to the action of the drug, since a similar effect was caused by Ringer's solution. The olfactory end organs are as chemoreceptors with a highly developed sensitivity obviously very susceptible to alterations in the ionic environment. Under physiological conditions only minute quantities of foreign substances reach the olfactory epithelium and the electrolyte equilibrium is kept constant by the secretory activity of the glands. When applying for instance Ringer's solution on the mucosa the ionic balance across the excitable membranes of the receptors is altered, and this change presumably leads to a functional disturbance in the epithelium. Alternatively it may be assumed that the application of a solution on the mucosa causes an increased activity in Bowman's glands

(see HOPKINS, 1926) and that the reduction of the response is due to the epithelium being covered by a thick layer of mucus, which to a certain extent prevents the stimulating particles from reaching the receptors. Anyhow, the response of the epithelium is not abolished by cocaine in a concentration sufficiently high to block the activity in the olfactory nerve fibres. This finding seems to support the conclusion that the origin of the potential is located in the sensory elements.

Additional evidence for this hypothesis was achieved in the experiments in which the olfactory nerve was antidromically stimulated. Furthermore the results of these experiments indicate that the olfactory end organs cannot be activated by antidromically arriving impulses. It might be argued that the impulse conduction may have been blocked at the axon-soma boundary and that the impulses in such a case would not reach the receptors. An alternative explanation is that the impulses were actually conducted up to the receptors but failed to invade them. It is known that there are fundamental differences between the electrical response of receptors and that of nerve fibres. The sensory end organs further appear to be functionally independent of their nerve fibres, as revealed by the fact that they preserve their excitability almost unchanged even after the nerves have been rendered completely inexcitable by the action of local anaesthetics. These differences indicate that the excitatory processes in the receptors differ from those in the axons. It may be assumed that a slow sustained potential cannot be evoked in the sensory terminals by antidromically conducted spike potentials in the nerve fibres and that consequently the excitation can be transmitted only from the receptors to the axons and not in the reversed direction. If this assumption is true, such a failure of the impulses to invade the receptors would explain why the response to olfactory stimulation was not blocked, when the olfactory nerves were electrically stimulated. Recently it has been reported (EYZAGUIRRE and KUFFLER, 1955 b; KUFFLER and EYZAGUIRRE, 1955) that the generator action in the dendritic terminals of the lobster's stretch receptor organ persists during antidromic stimulation of the axon.

As a direct consequence of the inference that the slow potential originates in the olfactory receptors, the question arose as to

which part of the primary olfactory neuron is to be identified as the sensory element. For many reasons it appears most likely that the olfactory hairs are the site of reception, although it cannot be excluded that the cell body proper or the peripheral process is sensitive to the stimulating agent. However, it is difficult to explain the gradual diminution of the potential when recording from increasing depth in the epithelium, if it is assumed that the soma of the olfactory neuron or the peripheral extension is the sensory part of the cell. The results indicate that the response originates in superficial structures which may be assumed as identical with the olfactory hairs.

In this connection it should be pointed out that there are reasons to presume that the olfactory hairs are the equivalents to the outer segments of retinal cells, while the peripheral extensions seem to correspond to the inner segments. This hypothesis is partly based on the fact that the outer limiting membrane in the olfactory epithelium corresponds to *membrana limitans externa* in the eye (see *e.g.* v. BRUNN, 1880). It may further be mentioned that the peripheral processes of the olfactory neurons contain a great number of mitochondria of the same ultrastructure (BLOOM, 1954) as those found in the inner segments of retinal cells (SJÖSTRAND, 1953).

From many points of view there are striking similarities between the olfactory epithelium and the retina, especially that of certain evertebrates. For instance the retina of *Eledone moschata* consists of a single layer of visual cells, from which fine nerve fibres pass to the optic ganglion which lies separated from the retina (see *e.g.* GRENACHER, 1886). The response obtained when illuminating the eye of *Eledone* is a slow negative monophasic potential, which is almost identical in shape and time course with the response evoked by olfactory stimulation of the nasal mucosa. FRÖHLICH (1914) assumed that the response in the eye of *Eledone* came from the visual cells. A similar potential is also obtained from the eye of the water beetle and BERNHARD (1942) showed that it originates in the receptors. The resemblance between the response recorded from the olfactory epithelium and that obtained from evertebrate retinas indicates that the processes associated with the excitation of the sensory cells of

these two types of sense organs are of similar nature. The slow potential of the evertebrate retina represents the photochemical processes which are elicited in the visual cells by light, while the response of the olfactory epithelium in all probability is elicited by reactions between substances in the olfactory receptors and the particles of the stimulating agent. The electrical response of the retina is usually called the electroretinogram, the ERG. In view of the structural and functional similarities between the retina and the olfactory sense organ the slow response of the olfactory epithelium may be called the electro-olfactogram, the EOG.

PART II

The Characteristics of the Response

A. *Relation between amplitude of response and strength of stimulus*

The increase of the amplitude of the response with increased intensity of the stimulus was studied in a series of experiments in which the preparation was stimulated with butanol of different concentrations. The volume of the stimulating air puff was usually 0.5 cc and the stimulation was always carried out in the order from lower to higher stimulus intensities. Although stimulation with 0.1 M butanol did not elicit the maximum response, higher stimulus intensities were avoided since it was found that strong stimulation often caused damage to the epithelium as revealed by a reduction of the excitability of the preparation.

The result of an experiment in this series is illustrated in Fig. 9. Record A shows the response evoked with purified air. The test bottle (k in Fig. 2) containing the stimulating agent was in this case replaced by a charcoal filter through which the air passed. Although the air was filtered three times and the outblow pipette carefully cleaned a small response was evoked. However, this result does not imply that the olfactory receptors can be mechanically stimulated but rather illustrates the difficulties encountered in removing all traces of odorous material from the expelled air.

Record 9 B shows the response to stimulation with a highly diluted butanol solution (0.001 M), the odour of which was just perceptible to the human nose. When 0.5 cc of this faintly odorized air was blown on to the nasal mucosa a response was evoked amounting to approximately 0.5 mV. When the stimulus intensity was further increased, the amplitude of the response increased (records C—F), and with 0.1 M butanol it reached 3.5 mV (F). Potentials amounting to 7—8 mV were often seen when still higher stimulus intensities or greater volumes of odorized air were used.

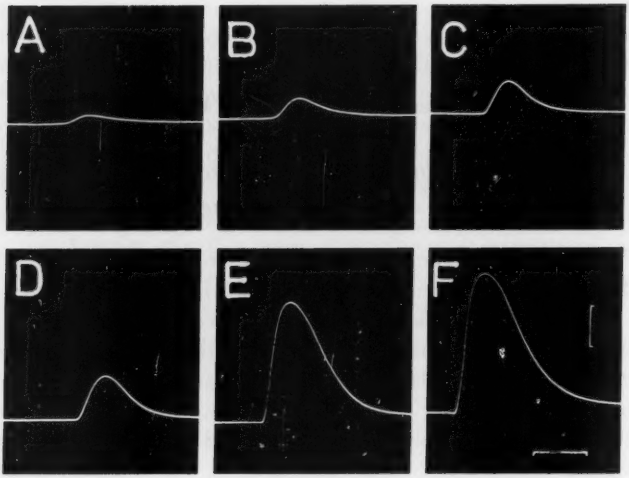

Fig. 9. Increase of amplitude of potential at increase of stimulus strength. Records of responses evoked by stimulation: A, with purified air; B—F, with butanol; B, 0.001 M; C, 0.005 M; D, 0.01 M; E, 0.05 M; F, 0.1 M. Volume of air 0.5 cc. Vertical line in F 1 mV. Time bar 2 sec.

In section D in this chapter a detailed description will be given of the changes in the shape of the response when the stimulus intensity is varied. In this connection it should only be mentioned that there occurred a broadening of the potential, when the stimulus strength was increased. As illustrated by the records in Fig. 9 this change is mainly due to an increase in the decay time of the response.

The relation between the amplitude of the response and the stimulus strength is further illustrated in Fig. 10. The curve is based on the mean values of 10 experiments and is obtained by plotting the amplitudes of the responses in per cent of the maximum response recorded in each series of stimulations against stimulus strengths. As shown by the curve the amplitude increased nearly 10 times when the stimulus strength was increased from 0.001 M to 0.1 M. The curve illustrates that the steepest rise in amplitude occurred at an increase of the stimulus strength from 0.005 M to 0.05 M.

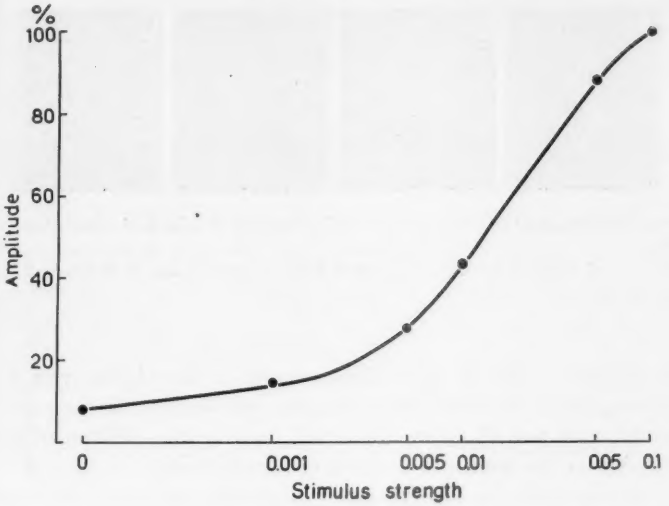

Fig. 10. Relation between amplitude of potential and stimulus strength. Ordinate: amplitude in per cent of response to 0.1 M butanol. Abscissae: stimulus strength; zero, purified air. Stimulus: butanol. Volume of stimulating air 0.5 cc. The stimulus strengths in this and the following curves are given in units corresponding to the concentrations of the solutions used for odorizing the stimulating air.

B. *Relation between amplitude of response and volume of stimulating air*

The changes in amplitude of the response with changes of the volume of the stimulating air were analysed in experiments in which the preparation was stimulated with different volumes of air of equal odour intensity. Alterations of the volume were achieved as described in Methods, and the following volumes were generally used: 0.1 cc, 0.25 cc, 0.5 cc and 1 cc. These figures are only approximative, owing to the difficulties encountered in estimating the exact volume of air expelled from the pipette. The tip of the pipette was usually kept 5 mm from the surface of the epithelium. If this distance was exceeded, stimulation with 0.1 cc of the odorized air usually failed to evoke any response. It was noticed - particularly when smaller volumes than 0.5 cc

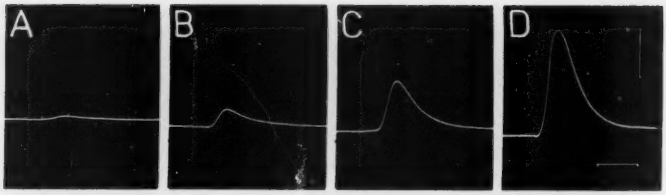

Fig. 11. Increase of amplitude of potential at increase of volume of stimulating air. Records of responses evoked by stimulation: A, with 0.1 cc; B, 0.25 cc; C, 0.5 cc; D, 1 cc of odorized air. Stimulus: 0.01 M butanol. Vertical line in D 1 mV. Time bar 2 sec.

were used - that a slight displacement of the pipette was accompanied by a marked change in the amplitude of the response. Great care was therefore taken not to alter the position of the pipette in the course of a series of stimulations.

If this precaution was taken only small differences were obtained between the results from different series of stimulations in one and the same preparation, whereas the results varied a great deal from one preparation to another. These differences were apparently due to the fact that the tip of the pipette was not kept at the same distance from the surface of the epithelium.

The records in Fig. 11 show the responses obtained in one of the experiments, in which the preparation was stimulated with four different volumes of odorized air (0.01 M butanol). Record A shows the small response evoked by stimulation with 0.1 cc of the gas. When the volume was increased the amplitude of the potential became successively higher, as shown by records B, C and D.

The relation between the amplitude of the response and the volume of the stimulating air is illustrated in Fig. 12. The curve represents the mean values of 6 series of stimulations in one preparation. It is obtained by plotting the amplitudes in per cent of the greatest response recorded in each series against the volumes of the stimulating air. The shape of the curve illustrates the rapid increase of the potential at an increase of the air volume from 0.25 cc to 1 cc. As also seen, the response to stimulation with 0.1 cc of the odorized air amounted only to about 5

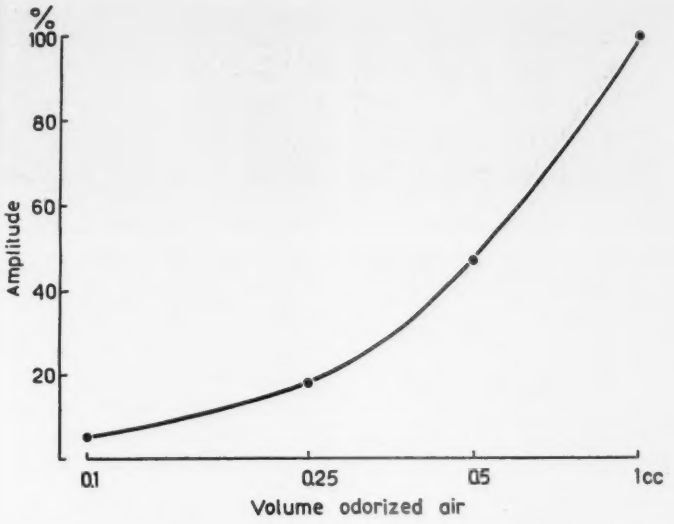

Fig. 12. Relation between amplitude of potential and volume of stimulating air. Ordinate: amplitude in per cent of response to 1 cc. Abscissae: volume of stimulating air. Stimulus: 0.01 M butanol.

per cent of that obtained at stimulation with 1 cc of the same gas. The latter response did not represent the maximum; still greater potentials were recorded when volumes greater than 1 cc were used. It should be pointed out that the curve in Fig. 12 illustrates the relationship between the amplitude of the response and the volume of the stimulating air at one particular stimulus strength. When higher or lower stimulus strengths were used, curves of different shapes were obtained.

C. *Response to stimulation with a constant amount of odorous material*

In investigations on different types of eyes (see *e.g.* HARTLINE, 1928) it has been established that the electrical reaction of the retina to illumination obeys within certain limits the BUNSEN-

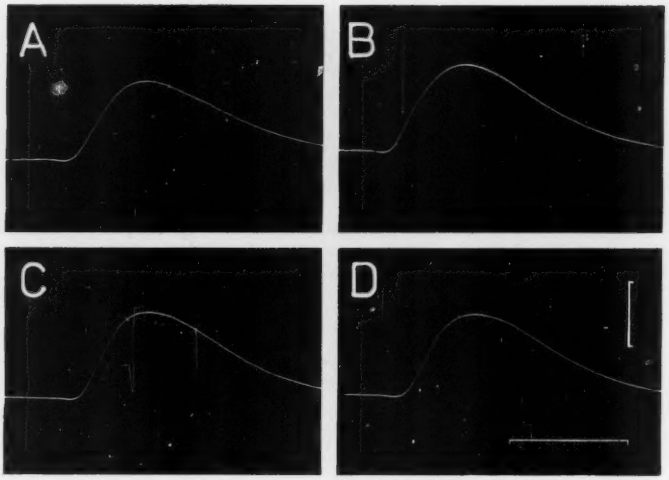

Fig. 13. Stimulation with equal amounts of odorous material. Records of responses to stimulation with different volumes of air of different stimulus strengths, the product of volume and stimulus strength being kept constant: A, 1 cc of 0.005 M; B, 0.5 cc of 0.01 M; C, 0.2 cc of 0.025 M; D, 0.1 cc of 0.05 M butanol. Vertical line in D 2 mV. Time bar 2 sec.

ROSCOE law, according to which a constant amount of energy ($I \times t = C$) is required for the production of a constant photochemical effect. The experiments to be described in this section were carried out in order to test whether, in analogy with the law for photochemical reactions, a constant amount of odorous material presented to the olfactory epithelium in air puffs of different durations would evoke responses of equal amplitudes. Since it was found difficult to alter the duration of the stimulation in a well defined manner, the volume of the expelled air was varied, whereas the duration of the stimulation was kept constant. By altering the strength of the stimulus so as to keep the product of volume and intensity constant, approximately equal amounts of odorous material were blown on to the nasal mucosa at each stimulation. For practical reasons the alterations of the volume had to be limited within the range of 0.1 cc to 1 cc.

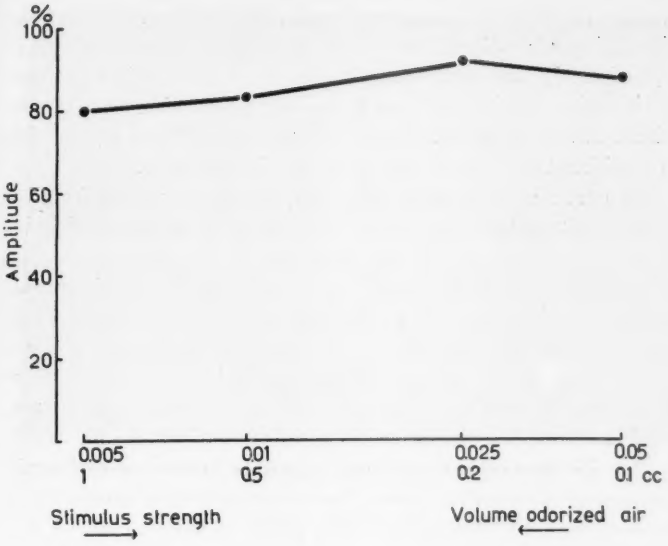

Fig. 14. Relation between the amplitude of the response (ordinate) and the strength and volume of the stimulating air (abscissae) on stimulation with a constant amount of odorous material. Mean values of 15 series of measurements, the maximum response in each series taken as 100 per cent. The curve does not reach the 100 per cent value, owing to the fact that the greatest responses were obtained at different stimulus strengths (and volumes of air) in different series.

Smaller volumes than 0.1 cc could not be administered with reasonable accuracy. Greater volumes than 1 cc were avoided since the stimulus strength then had to be lowered (in order to keep the product of volume and stimulus strength constant) to such a degree that even a slight contamination of the air in the test bottle would greatly influence the results. It could further be presumed that a considerable dispersion of the odorous material would occur if great volumes were used.

The records in Fig. 13 show the responses obtained in one of these experiments, in which the epithelium was stimulated with four different volumes of air odorized with butanol of different concentrations. Record A shows the response to stimulation with 0.005 M butanol, the volume of the air puff being 1 cc. When the

stimulus intensity was increased to 0.01 M and the volume of the stimulating air reduced to 0.5 cc a response was obtained of approximately the same amplitude (B). With further increase of the stimulus intensity and corresponding reduction of the volume of the air puff responses were recorded (C and D) showing no significant difference from that seen in record A.

The curve in Fig. 14 is based on the mean values from 15 series of stimulations and is obtained by plotting the amplitudes of responses in per cent of the greatest potential obtained in each series against stimulus strengths and volumes of stimulating air (in opposite directions on the abscissae). In spite of the obvious sources of error involved in the experiments the results indicate that a constant amount of odorous material evokes responses of approximately constant amplitudes even when the odorous material is distributed within different volumes of air. As illustrated in Fig. 14 the responses to high stimulus intensities and small volumes of odorized air were, however, somewhat greater than those evoked by stimulation with great volumes of air of low intensity.

Although the amplitude of the response varied only slightly in these experiments, it is obvious that the inversely proportional relationship between the strength of the stimulus and the volume of the stimulating air exists only within a limited range of variations of these variables.

D. *Changes in shape of response with changes of stimulus strength*

In the experiments described in section A it was observed that the responses evoked at varying stimulus strengths differed from each other not only in amplitude but also in shape. In order to study these differences experiments were carried out, in which the gain of the amplifier was adjusted so that responses of approximately the same amplitudes were obtained irrespective of the stimulus strength. The recordings were made with high film speed. For the detailed account of the shape of the response it was found convenient to describe the rising phase, the intermediate phase and the falling phase of the potential separately.

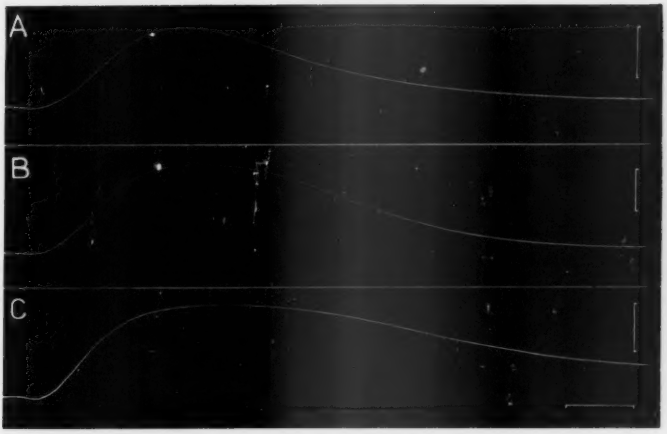

Fig. 15. Changes in shape of potential at increase of stimulus strength. Records of responses evoked by stimulation: A, with 0.001 M; B, 0.01 M; C, 0.1 M butanol. Volume of air 1 cc. Vertical line in A 1 mV, in B 1.5 mV, in C 4 mV. Time bar 0.5 sec.

The data given below on the characteristics of the different phases of the potential are valid only for responses obtained under the experimental conditions described. This fact is emphasized since it was observed that although the general character of the response remained unchanged, alterations of the "waveform" of the stimulation were followed by changes in the shape of the response.

I. *Changes in the rising phase.* Owing to the slow rise of the potential from the base-line as well as to the asymptotical approach towards the crest, it was difficult to determine exactly when the potential started and when it reached its maximum. The rising phase has therefore been defined as the rise of the potential from 10 to 90 per cent of the maximum amplitude. It should be mentioned that this corresponds to the usual definition of the rise of an electrical signal.

As illustrated by the records in Fig. 15 variations in stimulus strength were not accompanied by any marked changes in the features of the rising phase. A careful examination of the records, however, reveals that the rise of the potential evoked at a high

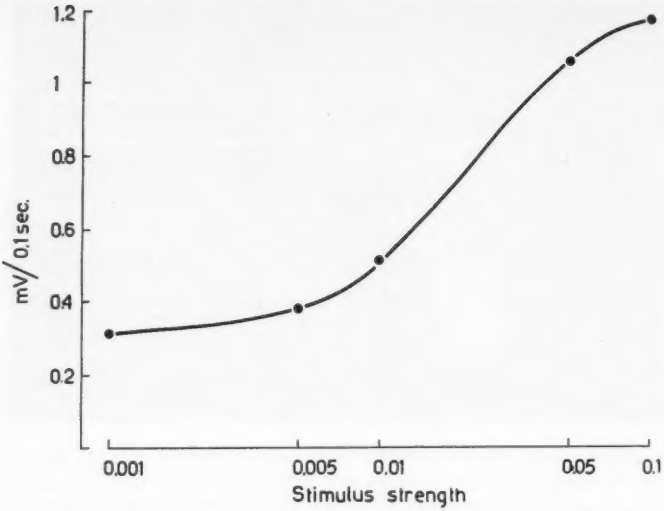

Fig. 16. Relation between rate of rise of potential (ordinate) and stimulus strength (abscissae). Stimulus: butanol. Volume of air 1 cc.

stimulus intensity (C) is somewhat steeper than that of the response at low stimulus strength (A).

The potential produced by a weak stimulation rises from 10 to 90 per cent of its maximum amplitude in approximately 0.6 sec, whereas the corresponding time of the response evoked by a strong stimulation is only slightly shorter.

The small variations in rise time of the response implied that the relation between the rate of rise of the potential and the stimulus strength must be similar to that between the height of the response and the stimulus intensity.

Fig. 16 shows the curve obtained by plotting the average rate of rise of the potential in mV/0.1 sec against stimulus strength. As seen, there is a striking resemblance between this curve and that illustrating the relation between the amplitude of the response and the stimulus intensity (Fig. 10). The curve in Fig. 16 shows that the rate of rise of the potential increased from 0.3 mV/0.1 sec at low stimulus strength to about 1.2 mV/0.1 sec on

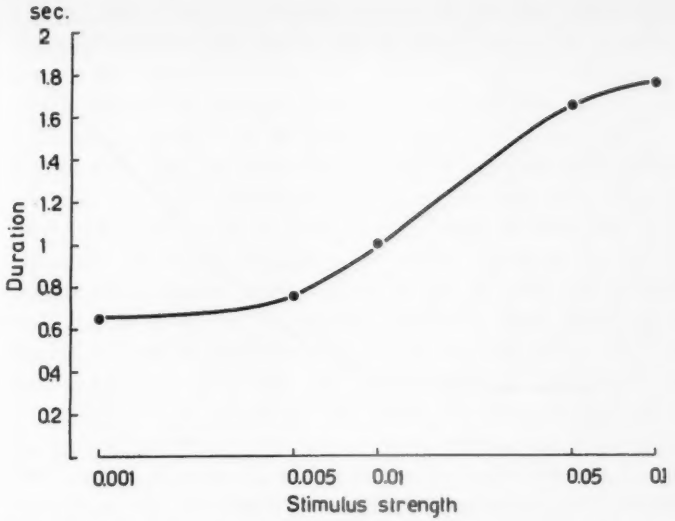

Fig. 17. Relation between duration of intermediate phase of potential (ordinate) and stimulus strength (abscissae). Stimulus: butanol. Volume of air 1 cc.

strong stimulation, the most marked change occurring in the same range of stimulus strength within which the curve relating the amplitude of the response to the stimulus intensity had its steepest rise.

The curves in Fig. 16—18 are based on mean values from 5 experiments in which the preparations were stimulated with butanol of different stimulus intensities. The volume of the stimulating air in these experiments was 1 cc.

II. *Changes in the intermediate phase.* This phase represents the crest of the response and has been defined as the part of the potential falling between the two 90 per cent values of the maximum amplitude. As illustrated by the records in Fig. 15 an increase of the stimulus strength is accompanied by a marked change in the shape of the intermediate phase. At low stimulus intensity (A) the fall of the potential starts without delay after the peak has been reached. At high intensity (C) the potential is maintained at the peak level for a comparatively longer time.

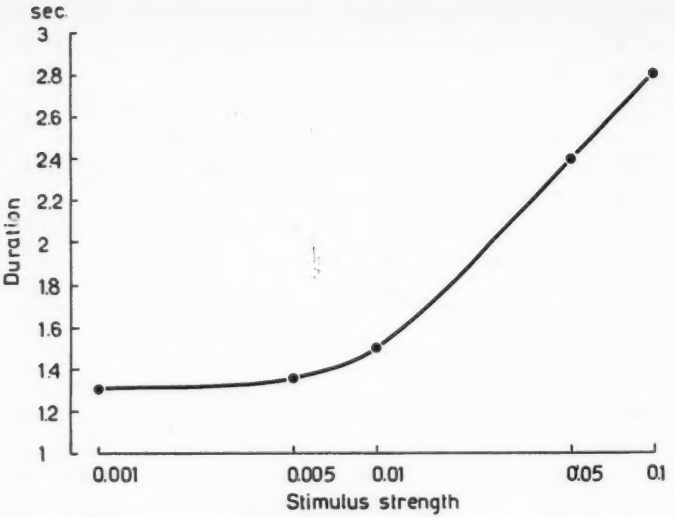

Fig. 18. Relation between duration of decay of potential (ordinate) and stimulus strength (abscissae). Stimulus: butanol. Volume of air 1 cc. See text.

This change leads to a broadening of the crest of the potential and consequently to an increased duration of the intermediate phase.

The relation between the duration of the intermediate phase and the stimulus strength is illustrated in Fig. 17. It will be seen that the duration increases from about 0.7 sec at low stimulus intensity to nearly 2 sec at high intensity, the most marked change occurring in the range of medium stimulus strengths.

III. *Changes in the falling phase.* The records in Fig. 15 illustrate that the greatest changes in the shape of the response with altered stimulus strength occur in the falling phase.

At low stimulus intensity (A) the falling phase of the response consists of an initial steep slope followed by a slow decline towards base-line, whereas the same phase of the response to strong stimulation (C) is characterized by a considerably longer duration.

Owing to the slow approach of the potential towards zero it was difficult to determine the total duration of the falling phase,

and the decay time was therefore taken as the time of fall of the potential from 90 per cent of the maximum amplitude to 30 per cent of this value.

The relation between the decay time and the stimulus strength is illustrated in Fig. 18. As shown by the curve it took about 1.3 sec for the potential to return to the 30 per cent level at low stimulus intensity, whereas at high intensities this level was not reached until after nearly 3 sec. It will further be seen that there occurred only a slight change in the decay time at an increase of the stimulus strength from 0.001 M to 0.01 M while the increase from 0.01 M to 0.1 M was accompanied by a steep increment of the time of fall. It should be pointed out that the curve in Fig. 18 does not give a true picture of the changes in the duration of the falling phase since, as mentioned above, the time of the final 30 per cent decay is not included; a curve relating the total decay time to the stimulus strength would presumably have a steeper slope than that seen in Fig. 18. The rate at which the potential declined from 90 per cent of the maximum amplitude to the 30 per cent level was measured as the fall in mV/0.1 sec. It was found that the potential evoked at low stimulus strength declined at an average rate of about 0.15 mV/0.1 sec. However, contrary to the rate of rise, the rate of fall changed only slightly at an increase of the stimulus strength. Due to the evident errors involved in these measurements the curve relating the rate of decay to the stimulus strength is not given.

E. *Relation between shape of response and "waveform" of stimulus*

The time course of the electrical response of the olfactory epithelium to odour stimulation can be assumed to depend on the number of odorous particles which impinge upon the surface of the epithelium per unit time. Since the flow of the stimulating air current was not likely to be constant throughout the duration of the stimulation, the amount of odorous material carried to the epithelium per unit time could not be presumed to be constant.

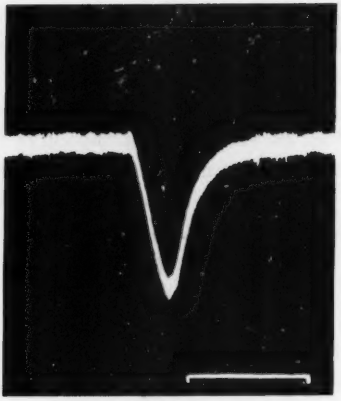

Fig. 19. »Waveform» of stimulus. Record of microphone response to the stimulating air puff. Time bar 2 sec. Full description in text.

Attempts were therefore made to examine the dynamical characteristics of the stimulating air puff in order to analyse the relation between the shape of the response and the "waveform" of the stimulus.

Owing to the small volume of the air puff there appeared to be no reliable method to measure the rate at which the air passed out of the pipette. The procedure finally adopted to study the air flow in the course of one single stimulation was to record the pressure changes induced by the air current. This was carried out by replacing the preparation with a sensitive crystal microphone and recording the pressure changes by means of a direct coupled amplifier connected to the oscillograph. In order to obtain a faithful recording of the pressure changes induced by the air current, the time constant of the microphone chosen had to be as long as possible, *i.e.* the capacitance and leakage resistance of the microphone had to be high. Further, it was necessary to make the connection to the amplifier through an electrometer stage. The total time constant of this stage and of the microphone was approximately 4 sec. Since the duration of the air puff was about 0.9 sec the time constant was apparently too short to allow of an undistorted recording. No attempts were made to determine

the linearity of the response of the microphone to pressure changes, owing to the obvious difficulties encountered in performing such a measurement.

The shape of the microphone response to a puff of air (1 cc) was found to be very like that of the response evoked by olfactory stimulation of the epithelium. The microphone response was characterized (Fig. 19) by a fast rising phase followed by a slow return towards base-line. A detailed comparison between the time course of the response of the microphone and that of the potential recorded from the epithelium cannot, however, be made owing to the uncertainties involved in measuring the pressure changes induced by the air puff. The striking resemblance between the two types of responses implies, however, that the "waveform" of the stimulus is of decisive importance for the shape of the response evoked in the olfactory epithelium.

F. *Responses evoked by different types of odorous substances*

In the course of some experiments, in which the responses to other substances than butanol were studied, it was observed that the features of these responses were not identical with those of the response to butanol. No systematic study of these differences and their relation to the physicochemical properties of the stimulating substances has been made in the present investigation. Some preliminary experiments will, however, be reported, which were carried out in order merely to examine whether the differences also appeared when different substances of equal odour intensities were used. For this purpose amyl acetate and oil of cloves were chosen and the concentrations of these substances were adjusted so that the responses elicited were of approximately the same amplitude as the response evoked by stimulation with 0.05 M butanol.

The records in Fig. 20 show the potentials set up by stimulation with amyl acetate (A), butanol (B), and oil of cloves (C). It is seen that the rising phases of the responses to these three substances do not differ significantly from each other, whereas

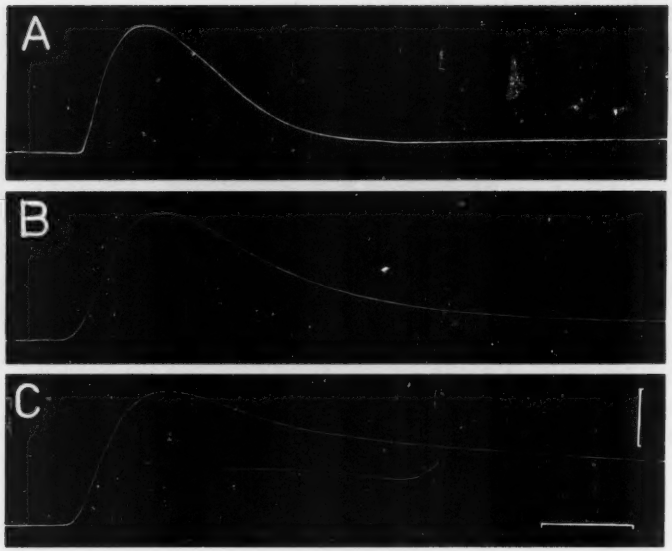

Fig. 20. Responses to different types of odorous stimuli. A, to amyl acetate; B, to butanol (0.05 M); C, to oil of cloves. Volume of stimulating air 1 cc. Vertical line in C 1 mV. Time bar 1 sec.

there are marked differences between the falling phases. As illustrated by record A the response to amyl acetate has a fast rising phase and a rapid decline from the crest towards zero. The duration of the response is considerably shorter than the corresponding time for the response to butanol or to oil of cloves. This difference is mainly due to the different rates at which the potentials decay. It will, however, also be noticed that the response to amyl acetate rises somewhat faster than that to butanol or to oil of cloves. As illustrated by record C the duration of the response to oil of cloves is remarkably long owing to the slow return of the potential towards base-line.

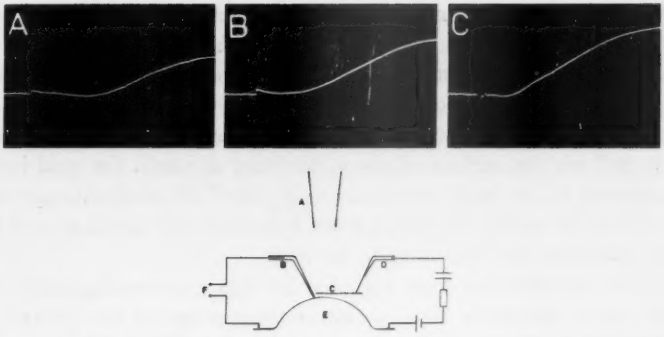

Fig. 21. Latency of response. Records of rising phase of response to stimulation: A, with 0.001 M; B, 0.01 M; C, 0.1 M butanol. Volume of air 1 cc. Time marks 0.1 sec. Schematic diagram below records shows the experimental arrangement: A, outblow pipette; B, recording electrode; C, strip of gold leaf; D, platinum wire fixed in micromanipulator; E, *eminentia olfactoria;* F, input to amplifier. Full description in text.

G. *Latency of response*

If the theory (see section G in Part III) is accepted that stimulation of the olfactory end organs requires contact to be established between the particles of the odorous substance and the epithelium, the latency of the response should be measured from the moment when the stimulating particles impinge upon the receptors.

A great difficulty encountered in the measurements of the latency of the response was to determine at what moment the stimulating air current reached the surface of the epithelium. The method finally adopted was the following (see schematic diagram in Fig. 21).

A small strip of gold leaf (3×0.5 mm, thickness 1—4 μ) was mounted on the end of a platinum wire (tip diameter 0.1 mm) fixed in a micromanipulator. The gold leaf could thus be brought into a position 50—100 μ above the surface of the epithelium. The platinum wire was connected to one pole of a battery via a condensor and a resistance, the other pole of the battery being connected to the preparation. The electrode with which the response to the olfactory stimulation was recorded was placed in contact with the epithelium close to the gold leaf. When the

preparation was stimulated the air current brought the gold leaf into contact with the epithelium. At this moment the circuit was closed and a signal appeared on the record (Fig. 21 A—C). It should be pointed out that the artefact was not presumed to indicate exactly the moment when the front of the stimulating air puff hit the surface of the epithelium, although the gold leaf appeared to be bent instantaneously when the stimulation was applied. Therefore the values given below are only to be regarded as indicating the approximate latencies.

The amplification was adjusted so that responses of almost the same amplitude were obtained irrespective of the stimulus strength. As illustrated by the records A—C in Fig. 21 the latency shortened as the stimulus strength was increased. It was, however, difficult to determine when the potential started, and thus an additional source of error was introduced into the measurements. In order to reduce this, three readings were made from each record. The mean values obtained in this way from 12 experiments are presented in Fig. 22. As is seen from this curve the latency of the response varies from approximately 0.4 sec at low stimulus strength to about 0.2 sec at the highest stimulus intensity used in these experiments.

The *true* latency of the response is the time lag between the moment when the stimulating substance reaches the excitable membrane of the receptors and the start of the potential. Even if it were possible to determine exactly when the air current hits the surface of the epithelium, the true latency would not be obtained, since the olfactory epithelium is covered with a layer of mucus through which the stimulating substance has to pass before reaching the receptors. Since neither the thickness of the mucus separating the receptors from the air nor the rate at which the substance diffuses through the mucus is known, it is impossible to determine exactly when contact is established between the odorous particles and the receptors. The time lag caused by the passage through the mucus can, however, be presumed to be rather short since, according to HOPKINS (1926) the long olfactory hairs reach the surface of the mucus and are to a considerable part of their length almost directly exposed to the air. If this is the case the latency obtained by measuring the

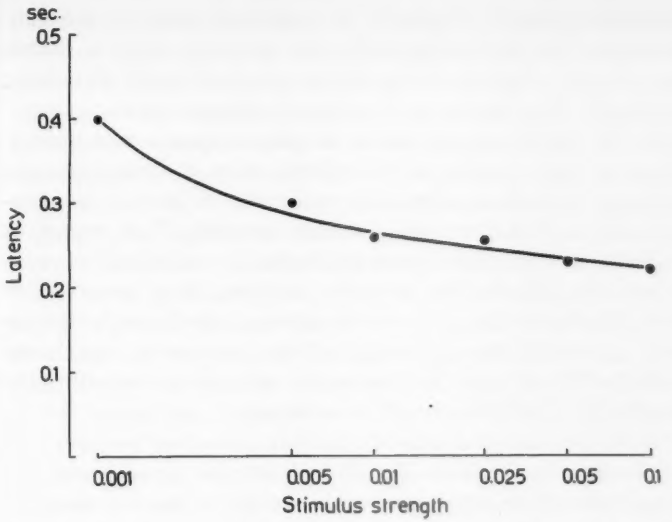

Fig. 22. Relation between latency of potential (ordinate) and stimulus strength (abscissae). Stimulus: butanol. Volume of air 1 cc.

time from the moment when the air reaches the epithelium until the response starts would represent the true value.

In section F in Part III some experiments will be described in which it was found that latency measurements could be made in another way owing to the occurrence of a positive potential, which under certain experimental conditions preceded the response. The latencies then obtained were found to agree with those given above.

H. *Discussion*

In studies on the electrical discharge in the olfactory bulb of the hedgehog ADRIAN (1942) found that responses were also elicited by stimulation with what appeared to be odourless air. He therefore concluded that the olfactory receptors respond to mechanical stimulation as well. Later on this theory was rejected (ADRIAN, 1951), since the results of further experiments showed

that under normal conditions the olfactory receptors are only stimulated by odorous particles. On the other hand it cannot be excluded, as pointed out by ADRIAN, that a forcible air current can excite the receptors by bending the olfactory hairs.

In the present investigation a small response was usually obtained when purified air was blown on to the nasal mucosa, although particular precautions were taken to prevent the contamination of the air with odorous material. There seems to be little reason to assume that the potential was elicited by mechanical stimulation of the olfactory receptors, since there is very little likelihood that all traces of odorous substances in the air were removed. The appearance of the response to stimulation with purified air was therefore merely regarded as indicating the insufficiency of the purification procedures.

In the physiology of sense organs great attention has been paid to the relation between the strength of the stimulus and the magnitude of the effect evoked in sensory elements. Generally this relationship has been evaluated by analysis of the impulse discharge set up in the afferent fibres, though in some preparations it has been possible to examine the relation more directly. Certain evertebrate eyes have offered such a possibility and from several investigations (HARTLINE, 1928; BERNHARD, 1942) it has been established that the amplitude of the slow response elicited in the visual cells is within certain limits proportional to the logarithm of the intensity of illumination.

Owing to the inaccessibility of the olfactory nerve fibres it has hitherto been difficult to analyse the relation between the magnitude of the effect evoked in the olfactory sense organ and the odour intensity of the stimulating air. HASAMA (1934) tackled the problem by recording the response in the pyriform lobe in the rabbit and by using ZWAARDEMAKER'S olfactometer (ZWAARDEMAKER, 1895). He found that the amplitude of the potential rose steeply to a maximum as the stimulus strength was increased. Apart from the uncertainties involved in the method applied by HASAMA it does not appear likely that the response in the pyriform lobe faithfully reproduces the events in the olfactory sense organ.

The slow potential of the olfactory epithelium offers the oppor-

tunities of a quantitative analysis of the reactions of the olfactory receptors to odorous substances. The results of the measurements performed show that the amplitude of the potential is proportional to the logarithm of the stimulus intensity. The range of stimulus strength, within which the response of the olfactory epithelium increases in amplitude with increasing odour intensity, was found to be of the same magnitude as the corresponding range for the response of the evertebrate eye to light stimulus (see *e.g.* HARTLINE, 1928; BERNHARD, 1942; AUTRUM, 1950). Since, however, the response of the single olfactory receptor is probably graded, it is not possible to determine to what extent the increase is due to an increment of the potential in the individual receptors or to the recruitment of receptors. Irrespective of the mechanisms responsible for the quantitative changes of the response, the results indicate that the same general relationship between the magnitude of the effect and the strength of the stimulus is valid for the olfactory epithelium as for other sense organs.

Since the recorded response represents the summated activity of all excited receptors it gives little information concerning the time course of the electrical events in the individual receptors. The question to what degree the time characteristics of the response of the epithelium reflect the events in the individual receptors cannot be settled until recordings have been made from single units in the epithelium. Owing to the small dimensions of the primary olfactory neurons all attempts in this direction have hitherto been unsuccessful. Very little is known about the temporal course of the response in other types of receptors. GRAY and SATO (1953) have, however, succeeded in analysing the potential of the isolated Pacinian corpuscle. Their finding that the rise time of the response in this structure is almost constant within a wide range of stimulus strengths is of special interest in this connection. In view of this observation it would be reasonable to assume that the olfactory receptor exhibits similar characteristics. If this assumption is true, the constancy of the rise time of the response in the olfactory epithelium would depend on the small changes in the rise time of the response in the individual olfactory receptors.

The temporal course of the response shows that the excitatory processes in the olfactory epithelium considerably outlast the duration of the stimulating air puff. This finding indicates that the odorous particles are slowly removed from the nasal mucosa. Until the particles of the odorous substance are wiped out of the olfactory epithelium the receptors are subjected to a continuous stimulation. With an increase of the stimulus intensity a greater amount of odorous material impinges upon the mucosa, and consequently the removal of the substance will take a longer time. An increase of the stimulus strength therefore implies a stimulation not only of greater intensity but also of increased duration. This would explain why the decay time (Fig. 18) as well as the duration of the intermediate phase (Fig. 17) of the response is prolonged when the odour intensity of the stimulating air is increased.

Since according to PARKER (1922) the olfactory hairs are of lipoid nature it can be presumed that the lipoid solubility of the stimulating substance is of importance for the time course of the response. However, before the odorous particles reach the sensory hairs, they must pass through a layer of mucus and consequently water solubility would also be important. It can therefore be expected that due to different physical properties various types of odorous substances give responses of different time relations. As previously mentioned ADRIAN (1951) found that the potentials which were evoked in the olfactory bulb of the rabbit by stimulation with various substances exhibited different temporal characteristics. Thus it was found that esthers such as amyl acetate elicited a discharge characterized by a sudden onset and a rapid decrease, while substances with an "oily smell" gave a response which started slowly and ceased gradually. These differences were also seen in records obtained when the discharge to different substances of equal odour intensity was measured quantitatively with an integrator (MOZELL and PFAFFMANN, 1954). It has been discussed (ADRIAN, 1951, 1954) whether the different time relations are due to differences in solubility in water of the stimulating substances or to the different rates at which the odorous particles diffuse to varying regions of the folded mucosa.

The temporal characteristics of the responses evoked in the flat receptor sheet of the nasal mucosa in the frog by stimulation with amyl acetate and oil of cloves (Fig. 20) are almost identical with the time relations of the responses evoked in the bulb of the rabbit with corresponding substances (ADRIAN, 1951; MOZELL and PFAFFMANN, 1954). This finding indicates that the temporal differentiation in the bulb is not dependent on the different rates of diffusion of the odorous particles into the nasal cavities nor on the folding of the mucosa. It is more likely that the solubility of the stimulating substances in water and lipoids is the decisive factor. It may be presumed that water solubility facilitates the penetration of the odorous particles through the mucus, thus enabling a rapid initiation of the response. Odorous substances, which are more readily dissolved in lipoids than in water, are obviously bound to pass slowly through the mucus and to be gradually removed.

The different physical properties of the stimulating substances cannot, however, entirely explain the differences in time course between the responses. It is obvious that other factors also are of importance. Most odorous substances are unsaturated and there exists a certain relationship between the degree of unsaturation and the odour intensity. It is therefore likely that the chemical reactivity of the stimulating agent is partly responsible for the temporal course of the excitatory processes in the epithelium.

In the course of the analysis of the characteristics of the response the question arose as to whether the potential is of a composite nature. The smooth monophasic appearance of the response did not rule out such a possibility, all the more so as there are indications that the slow monophasic potentials of certain evertebrate eyes are made up of two components (THERMAN, 1940; BERNHARD, 1942). No serious attempts have been made in the present investigation to study this problem. Certain observations were made which undoubtedly favoured the view that the potential was built up of fractions which had different properties, but it must be emphasized that no well defined components were isolated.

PART III

Some Functional Properties of the Olfactory Epithelium

A. *Response to continuous stimulation*

The adaptation of the olfactory epithelium was studied in experiments in which the nasal mucosa was stimulated with a continuous flow of odorized air. Since the preparation was found to be rapidly exhausted by this type of stimulation, the velocity of the stimulating air stream was kept low (1 cc/sec). The duration of the stimulation was usually limited to 15 sec. In order to avoid deterioration of the epithelium and to allow the receptors to recover, the preparation was left unstimulated for at least 15 min after each stimulation. The gain of the amplifier was adjusted so that responses of approximately identical amplitudes were obtained irrespective of the strength of the stimulus.

It was found that the flow of odorized air evoked a potential which declined from its crest to a level which was maintained almost constant throughout the ensuing duration of the stimulation. The height of this phase of the response in relation to the crest amplitude was lower the higher the stimulus intensity. This is illustrated in Fig. 23, showing the responses obtained from three preparations, each of them subjected to a continuous stimulation lasting for 15 sec. Butanol was used as stimulating substance, the strength of the stimulus being 0.001 M (A), 0.01 M (B), and 0.1 M (C). The flow of the odorized air was 1 cc/sec. The records show that the responses differ from each other mainly as regards the relative height of the steady potential to which the response drops from the crest. At low stimulus intensity (A) the amplitude of this phase of the response amounts to about 70 per cent of the peak value, whereas at medium stimulus strength (B) the potential drops to about 50 per cent before the constant level is reached. At high stimulus intensity (C) the decline goes still further, and the potential falls to about 30 per cent of the maximum value. A close inspection of the records obtained at high stimulus intensity revealed that the

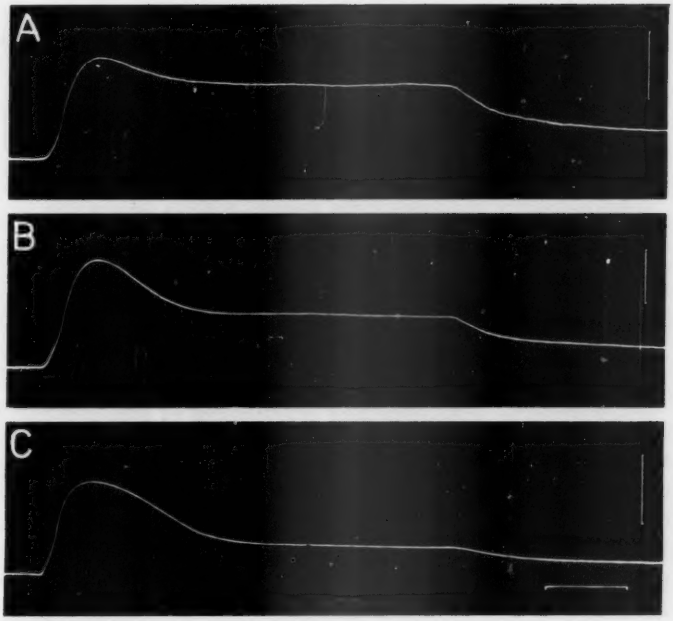

Fig. 23. Stimulation with a continuous flow of odorized air. Records of responses to stimulation: A, with 0.001 M; B, 0.01 M; C, 0.1 M butanol. Duration of stimulation 15 sec. Velocity of stimulating air stream 1 cc per sec. Vertical line in A 0.5 mV, in B 1 mV, in C 3 mV. Time bar 3 sec.

potential often did not remain constant at this level but showed a gradual decay. Thus it was observed that the amplitude of the response at the end of a stimulation of high intensity often amounted to less than 20 per cent of the crest value. The decline of the response to intense stimulation apparently revealed that the receptors were on the verge of exhaustion.

The records in Fig. 23 also illustrate the differences between the falling phases of the responses evoked at different stimulus strengths. On cessation of the stimulation the potential slowly returns towards base-line, the time of the decay being longer at high stimulus intensity than at low stimulus strength.

Similar changes in the falling phase were also found with

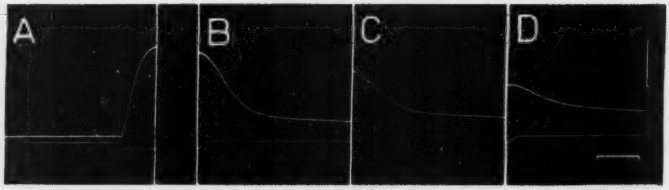

Fig. 24. Changes in falling phase of response with changes of duration of stimulation. A, rising phase of response. B—D falling phases of three responses (the rising phases of which were identical with that seen in A) to stimulations lasting for: 3 sec, (B); 5 sec, (C); 10 sec, (D). Stimulus: 0.01 M butanol. Velocity of stimulating air stream 1.5 cc per sec. Stimulation marked on lower beam. Vertical line in D 2 mV. Time bar 2 sec.

changes of the duration of the stimulation. This is illustrated by the records in Fig. 24 showing the responses obtained in one experiment in which the preparation was stimulated for periods of 3 sec, 5 sec, and 10 sec, equal stimulus strengths being used. Record A shows the rising phase, which was of identical shape and amplitude in the three responses, the falling phases of which are shown in B—D; the record in B shows the falling phase of the response elicited by stimulation for 3 sec, C for 5 sec, and D for 10 sec.

In several experiments the response evoked at low or medium stimulus strength did not remain at the constant potential level but increased slowly, and at the end of the stimulation the amplitude was even greater than the peak value. Since this type of response was most often seen in preparations which had been subjected to several stimulations it was not regarded as a normal type of response.

B. *Responses to repeated stimulations*

When the preparation was stimulated at short time intervals, the responses became successively lower, the reduction of the amplitude being related to the strength of the stimulus. The stimulations were so timed that each stimulation was applied

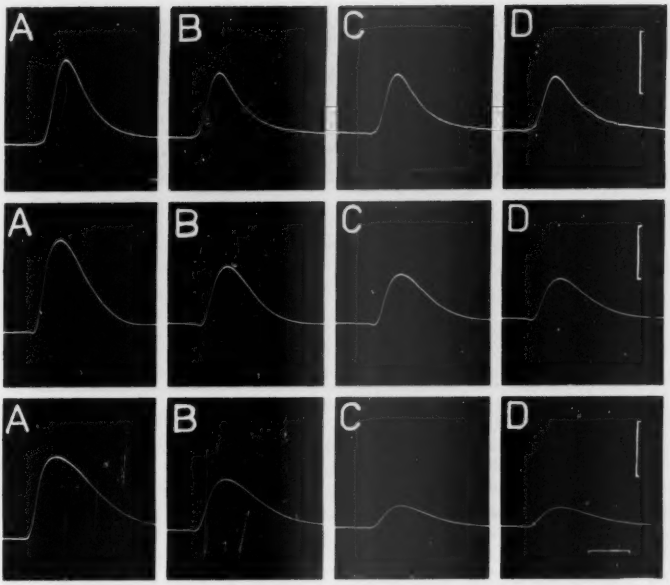

Fig. 25. Repeated stimulations. Responses to stimulation with: 0.001 M (upper row); 0.01 M (middle row); 0.1 M (lower row) butanol. A, response to first; B, to second; C, to fourth; D, to seventh stimulation in a series of successive stimulations. Stimulus interval: 10 sec. Vertical line in D: upper row, 1 mV; middle row, 2 mV; lower row, 5 mV. Volume of air 0.5 cc. Time bar 2 sec.

when the potential evoked by the preceding one had almost declined to base-line. Owing to the slow final approach of the potential to zero the intervals usually had to be kept as long as 10 sec.

The records in Fig. 25 show the responses obtained in three experiments, in which the preparations were stimulated with butanol. The records in the upper row were obtained on stimulation with 0.001 M, those in the middle row with 0.01 M, and those in the lowest row with 0.1 M butanol. The greatest change occurred initially as illustrated by the difference in amplitude between the response to the first (A) and the second stimulation (B). The reduction of the response in the course of the

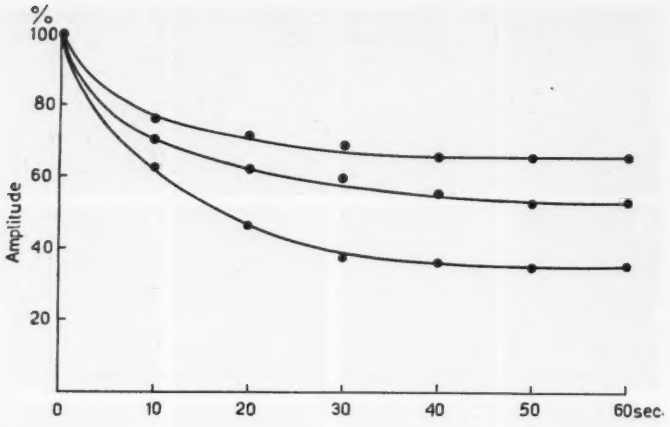

Fig. 26. Curves illustrating the diminution of response on repeated stimulations of the epithelium with: 0.001 M (upper curve); 0.01 M (middle curve); 0.1 M (lower curve) butanol. Stimulus interval: 10 sec. Ordinate: amplitude of response in per cent of first potential. Abscissae: time in sec. Volume of air 0.5 cc.

ensuing stimulations was, however, small at low stimulus strength (C and D in upper row), whereas at high stimulus intensity there was a marked diminution of the potential (C and D in lowest row). The time course of the decrease in height of the responses evoked at different stimulus strengths is illustrated in Fig. 26, each curve representing the mean values of 7 experiments.

The preparation was usually left unstimulated for 10 to 20 min after each series of stimulations in order to allow the epithelium to recover. If this precaution was not taken the decay of the amplitude of the potential was less pronounced. It may be added that in these series of experiments observations were made indicating that the potential was built up of two separate components with different properties; one being rapidly abolished by repeated stimulations, the other being resistant.

C. *Recovery*

In order to study the time course of the recovery of the epithelium after a stimulation, experiments were carried out in

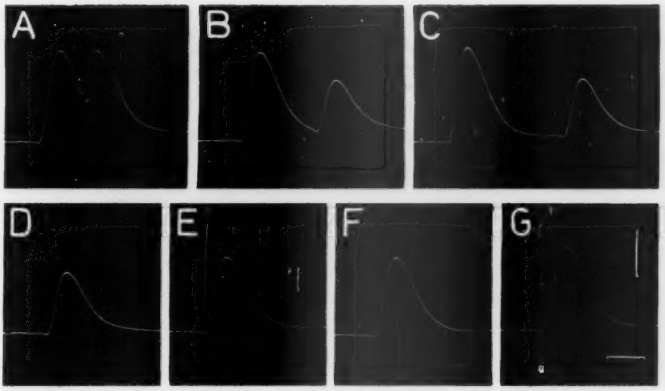

Fig. 27. Recovery. Records of responses to two successive stimulations of equal intensity, the second being applied at increasing intervals after the first. A—C: records of responses to first and second stimulation. Stimulus interval: A, 2 sec; B, 4 sec; C, 6 sec. The records D—G show the gradual increment of the response to the second stimulation at further increase of stimulus interval: D, 10 sec; E, 16 sec; F, 38 sec; G, 58 sec. (The response to the first stimulation in each case being identical with the first potential seen in C). Stimulus: 0.01 M butanol. Volume of stimulating air 0.5 cc. Vertical line in G 1 mV. Time bar 2 sec.

which a second stimulation was applied at different intervals after the first one. Both stimulations were of the same stimulus strength.

When the second stimulation was applied before the response to the first one had declined to zero, a potential was evoked, the amplitude of which was considerably smaller than that of the first response. As the interval between the two stimulations was lengthened, the second response became successively greater but did not amount to the amplitude of the first response until elicited after an interval of more than one minute. This time usually exceeded the period of decline of the first response to base-line.

The responses obtained in an experiment, in which 0.01 M butanol was used for stimulation, are shown in Fig. 27. Records A—C show the responses to the first and second stimulations, whereas D—G only show those to the second one, the response to the

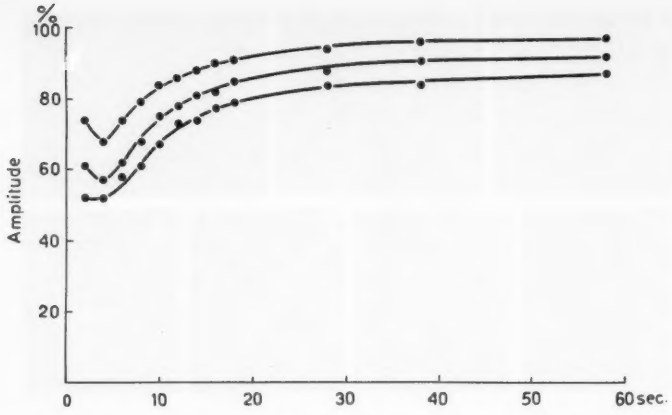

Fig. 28. Curves illustrating the relation between the amplitude of the second response and the time interval between first and second stimulation. Ordinate: amplitude of second response in per cent of amplitude of first potential. Abscissae: time interval between the two stimulations. Stimulus: 0.001 M (upper curve); 0.01 M (middle curve); 0.1 M (lower curve) butanol. Volume of air 0.5 cc.

first stimulation in each case being identical with the first response in record C. The intervals between the two stimulations were as follows: in A 2 sec, in B 4 sec, and in C 6 sec, the responses in D—G being elicited 10, 16, 38, and 58 sec after the first stimulations.

As illustrated in Fig. 27 the second response was small when elicited during the period of decay of the first potential. In C the interval between the two stimulations was sufficiently long (6 sec) to allow the first response to drop almost to zero before the second stimulation was applied, the response to which, however, only amounted to about 60 per cent of the amplitude of the preceding potential. At longer intervals the second response became successively greater, and when the second stimulation was applied 38 sec (F) after the first one a response was obtained, the amplitude of which reached about 90 per cent of the amplitude of the preceding potential.

The time course of recovery after stimulation with stimuli of different strengths is illustrated in Fig. 28. The curves were

obtained by plotting the amplitude of the second response in per cent of that of the first potential against the time interval between the two stimulations. The curves represent the results from experiments, in which 0.001 M (upper curve), 0.01 M (middle curve), and 0.1 M (lower curve) butanol were used as stimuli, each curve being based on mean values from 5 experiments.

As seen in Fig. 28 the relative amplitude of the second response is smaller the higher the strength of the stimulus. It will, moreover, be noted that the response elicited after an interval of 2 sec is slightly greater than that evoked 4 sec after the first stimulation, the difference being most marked at a low stimulus intensity. As seen in Fig. 27 A it is difficult to estimate the exact amplitude of the second response, when this is superimposed upon the steep slope of the falling phase of the first response. Therefore it cannot be excluded that the difference is due to errors of measurements, although the following explanation appears more likely. Since submaximal stimulus strengths were used, it is probable that the first stimulation failed to excite high threshold receptors but changed their excitability, so that the second stimulation when applied within a certain critical time interval activated them in addition to low threshold receptors. This view is supported by the fact that the difference between the amplitude of the response evoked 2 sec after a preceding potential and that elicited after 4 sec is smaller the higher the stimulus intensity.

The curves illustrate the rapid increase of the amplitude of the second response, when the interval between the two stimuli is increased from 5 to 20 sec, a further lengthening of the interval being accompanied by a less marked change in amplitude of the second response. It will further be seen that the recovery of the epithelium does not run parallel with the decay of the potential. At for instance 6 sec after the stimulation of the epithelium the potential has dropped almost to zero (Fig. 27 C) while the excitability of the epithelium at the same time is reduced to about 60 per cent, as revealed by the amplitude of the second response.

It should be mentioned that the recovery was more rapid after stimulation with amyl acetate than after stimulation with butanol

of equal odour intensity. This difference may possibly be related to the previously described comparatively rapid rate of decay of the response evoked by amyl acetate.

D. *Selective fatigue*

It is well known that olfactory fatigue is to a certain extent selective. Thus when the olfactory organ has become exhausted by strong olfactory stimulation with one substance so that the sensation of odour has disappeared, other odours can still be perceived. It has been assumed that this phenomenon indicates the existence of different types of receptors with specific sensitivities to different odours. Attempts have also been made to classify odorous substances on the basis of studies on selective fatigue in man (see *e.g.* ARONSOHN, 1886; DUNCAN and BEAN, 1950).

In order to study whether it was possible to reduce the sensitivity of the epithelium selectively to particular substances, a series of experiments was carried out in which the preparation was repeatedly stimulated with one substance until the receptors were on the verge of exhaustion. At this moment stimulation with a dissimilar type of odorous material was applied. In addition to the usual experimental arrangements, a second stimulus set-up was used, by which a rapid shift could be achieved from one type of stimulus to another. Before the beginning of the experiments the strengths of the two stimuli were adjusted so that the amplitudes of the responses were approximately equal. The experiments were performed as follows. The preparation was repeatedly stimulated with one substance, the conditioning stimulus, at intervals of 10 sec, until the response was reduced to about 30—40 per cent of the original amplitude. The repetitive stimulation was then discontinued and 10 sec later the preparation was stimulated with the second type of stimulus, the test stimulus. Since substances of comparatively high concentrations were used 5—6 successive stimulations were usually sufficient to produce a reduction of the response to the 30—40 per cent level.

In a first series of experiments butanol and amyl acetate were

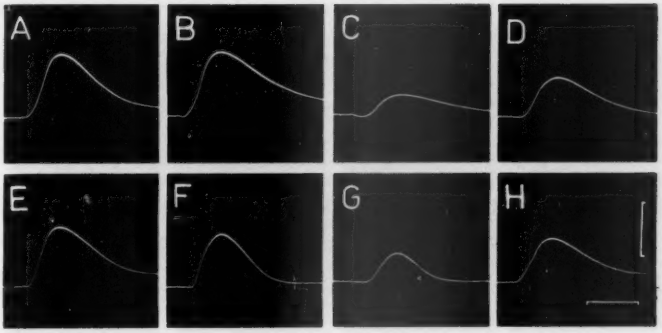

Fig. 29. Selective fatigue. Responses to stimulation with amyl acetate (A and D) and butanol (B and C). Records A and B obtained before, C and D after the preparation had been repeatedly stimulated with butanol. E—H another preparation. Responses to stimulation with butanol (E and H) and amyl acetate (F and G). Records E and F obtained before, G and H after the preparation had been repeatedly stimulated with amyl acetate. Vertical line in H 3 mV. Time bar 2 sec. See text.

chosen for the study of the interaction of substances belonging to different chemical classes. In a few experiments in this series the effect of oil of cloves and amyl acetate was also tried.

Records A—D in Fig. 29 illustrate the result obtained in one experiment, in which butanol was used as the conditioning stimulus and amyl acetate as the test stimulus. The responses to single stimulations with these two substances were of the same height (A and B). During the course of 6 successive stimulations with butanol, the amplitude of the response was reduced to the 30 per cent level (C). The subsequent test stimulus (amyl acetate) evoked a response (D), which was markedly greater than the last response to the conditioning stimulus (butanol), and which amounted to nearly 60 per cent of the amplitude of the unconditioned response (A).

Similar results were obtained when amyl acetate was used as conditioning stimulus and butanol as test stimulus. Owing to the rapid recovery after stimulation with amyl acetate, repetitive stimulation with shorter intervals had to be used in order to reduce the response to the 30—40 per cent level with 5—6

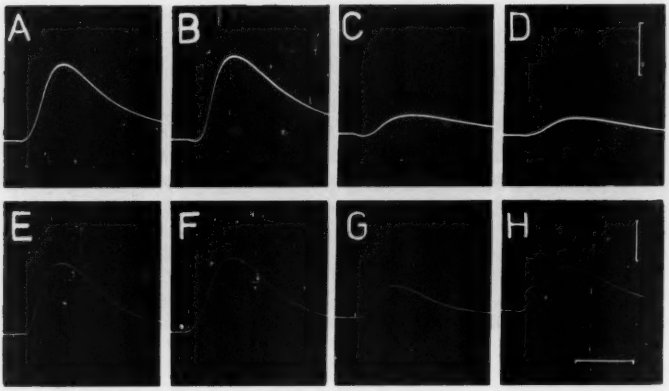

Fig. 30. Selective fatigue. Responses to stimulation with propanol (A and D) and butanol (B and C). Records A and B obtained before, C and D after the preparation had been repeatedly stimulated with butanol. E—H another preparation. Responses to stimulation with butanol (E and H) and propanol (F and G). Records E and F obtained before, G and H after the preparation had been repeatedly stimulated with propanol. Vertical line in D and H 2 mV. Time bar 2 sec. See text.

successive stimulations. Even if this was not obtained - as illustrated in records E—H in Fig. 29 - the response to the test stimulus was greater than that to the conditioning stimulus after the repetitive stimulation.

Almost identical results were obtained when the epithelium was stimulated with oil of cloves and amyl acetate regardless of which of these substances was used as conditioning stimulus.

In a second series of experiments butanol and propanol were chosen in order to study the interaction of substances of similar chemical constitution. In one of these experiments (Fig. 30 A—D) the repetitive stimulation with the conditioning stimulus (butanol) was accompanied by a reduction of the response to less than 30 per cent (C) of the original amplitude. The response to the subsequent test stimulus (propanol) was in this case found to be reduced to the same extent (D). These records illustrate one type of results rather often seen, another type being illustrated by records E—H in the same figure. In this latter experiment

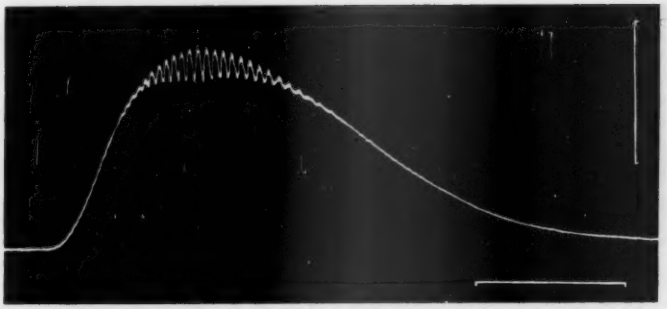

Fig. 31. Rhythmic waves superimposed upon the slow potential. Stimulus: amyl acetate. Volume of stimulating air 0.25 cc. Vertical line 5 mV. Time bar 1 sec.

propanol was used as conditioning stimulus and butanol as test stimulus. Before the preparation was repeatedly stimulated with propanol, the response to this substance (F) had almost the same amplitude as the response to butanol (E), whereas after the conditioning stimulation propanol (G) evoked a smaller response than butanol (H).

Owing to the great variability of the results, it was difficult to ascertain whether there was any significant difference or not between the results in this series of experiments and those obtained when substances of different chemical classes were used. The mean value of the relative reduction of the response to the test stimulus was therefore calculated from the results obtained in each series of experiments. It was then found that the reduction of the response to the test stimulus was greater in the second series of experiments than in the first one.

E. *Rhythmic waves*

In many experiments regular oscillations with a frequency of 15—25 per sec appeared superimposed upon the crest of the slow potential (Fig. 31). The oscillations arose at the end of the rising phase as small waves, which rapidly increased in amplitude and then faded out when the slow potential started to decline. They were generally not seen until some hours after the dissection

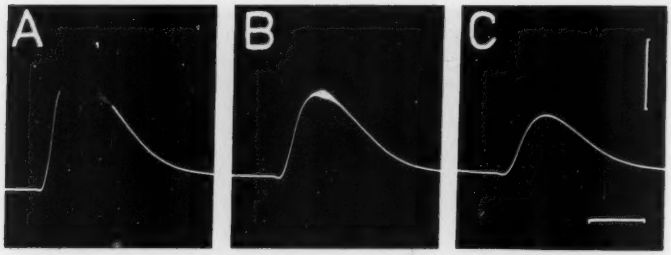

Fig. 32. Repetitive stimulation. Effect upon rhythmic waves. Records of responses set up by three successive stimulations with 0.1 M butanol. Stimulus interval: 10 sec. Vertical line in C 3 mV. Time bar 2 sec.

and were usually obtained only at high stimulus intensities. When the oscillations appeared at low stimulus strength, which occasionally happened, they increased in amplitude as well as in frequency with increasing stimulus intensity. In a few experiments the oscillations were also observed in quite fresh preparations. In one of these they suddenly appeared after the epithelium had been mechanically injured. When the preparation was repeatedly stimulated the waves rapidly decreased in amplitude and vanished (Fig. 32) but reappeared when the epithelium was allowed to recover for some minutes. The appearance of the waves in a preparation was often accompanied by a gradual diminution of the slow potential and they could usually be obtained only for a short time and then disappeared. When the preparation was stimulated with a continuous flow of odorized air the oscillations sometimes occurred as waxing and waning waves superimposed upon the sustained potential (Fig. 33).

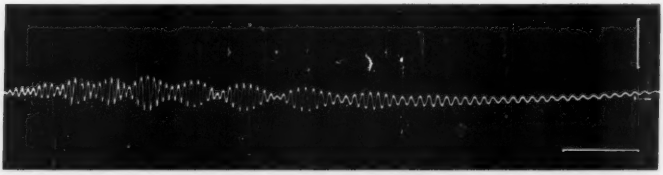

Fig. 33. Rhythmic waves evoked by continuous stimulation of the olfactory epithelium. Stimulus: 0.1 M butanol. Velocity of stimulating air stream 1 cc per sec. Vertical line 1 mV. Time bar 0.5 sec.

F. *The effect of increased humidity in the stimulating air*

In many experiments the response was preceded by a small positive deflection (see *e.g.* Fig. 5). This potential was usually seen only when the volume of the stimulating air exceeded 0.5 cc, or when the tip of the pipette was kept close to the surface of the epithelium. Since no relation was found between the amplitude of the positive deflection and the stimulus strength, it was assumed that this response did not arise due to stimulation of the olfactory receptors.

In the course of the investigation it was found that the amplitude of the positive deflection increased with increasing humidity of the stimulating air. The following arrangements were therefore made in order to study this effect more closely. The charcoal filter and the test bottle were kept in a tank with water, the temperature of which was varied. Except for the tip the outblow pipette was enclosed in a perspex tube, through which water of the same temperature as that in the bath was run. Since aqueous solutions of butanol were used for stimulation, an increase of the temperature of the bath caused an increase of the humidity of the stimulating air. Records A—D in Fig. 34 show that the amplitude of the positive deflection increased when the temperature of the bath was increased. When the butanol solution in the test bottle was replaced by a small piece of filter paper soaked in butanol and the stimulating air was kept dry, no positive dip appeared when the temperature was again increased (E—H).

It was therefore assumed that the positive deflection was evoked by stimulation of structures sensitive to changes in the humidity of the air. This assumption did not appear unreasonable, taking into consideration ZOTTERMAN'S (1949) discovery of the receptors in the frog's tongue which respond specifically to water. Further, it has to be noted that the ability of certain insects to distinguish differences in humidity of the air has been ascribed to specific humidity receptors on the antennae (see *e.g.* WIGGLESWORTH, 1941). However, further experiments, in which a similar response was obtained for instance from the surface of a muscle, brought definite evidence that the positive deflection

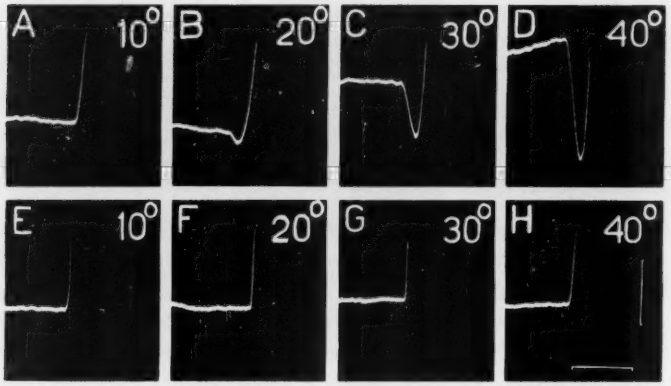

Fig. 34. The effect of increased humidity of the stimulating air. Records of initial phase of response to stimulation with butanol. Increase of humidity (A—D) obtained by increase of temperature of aqueous solution of the stimulating substance. E—H, increase of temperature of stimulating air when kept dry. Vertical line in H 200 μV. Time bar 2 sec. Full description in text.

recorded from the olfactory epithelium had to be regarded as an artefact.

It is not clear how this potential arises. Most likely the deflection is produced by positively charged water particles, carried by the stream of the stimulating air. Such an hypothesis agrees with observations reported by ZWAARDEMAKER *et al.* (see *e.g.* ZWAARDEMAKER, 1916; ZWAARDEMAKER, KNOOPS and VAN DER BIJL, 1916). They have found that a positive charge was carried by the water particles which were formed when water containing a small amount of an odorous substance was sprayed into the air.

If the positive deflection is induced by charged particles in the stimulating air, the start of the potential would indicate the moment when the air stream reaches the surface of the olfactory epithelium. Consequently the time interval between the start of the positive dip and that of the response to olfactory stimulation would give the latency of the response. This time interval was therefore measured in a series of experiments, in which the humidity of the stimulating air was kept high enough to make

the positive deflection appear. Owing to the slow onsets of the two potentials it was difficult to estimate with accuracy the interval between the beginning of the positive dip and the start of the response of the epithelium. In spite of the sources of error involved in these measurements the values obtained were found to agree with those given in section G in Part II.

G. *Studies on the mechanisms of olfactory stimulation*

A great number of theories have been advanced in order to explain the mechanisms of stimulation of the olfactory receptors. These theories, a few of which are based on experimental data, may be divided into two groups: the corpuscular theories and the radiation theories (see *e.g.* HENNING, 1924; MONCRIEFF, 1951).

According to the corpuscular theories the olfactory receptors are stimulated when particles of an odorous substance are brought into contact with the olfactory epithelium. Most of the authors who are of this opinion assume that the stimulation is due to the chemical action of the substance on the sensory cells, but it has also been suggested that the receptors are excited by molecular vibrations (see the review by MONCRIEFF, 1951).

According to the radiation theories the olfactory receptors may be regarded as distance receptors. No contact between the stimulating substance and the receptors is required, since the odorous material is assumed to emit radiation which stimulates the receptors in a way similar to that in which the visual cells are stimulated by light. A theory which has attracted great interest and caused much discussion has been advanced by BECK and MILES (1947). On the basis of observations on the behaviour of insects, these authors have suggested that the olfactory receptors emit infra-red radiation, which is absorbed by the molecules of the odorous substance. Owing to this absorption, energy is withdrawn from the receptors. The loss of energy is assumed to be the trigger mechanism which leads to stimulation of the receptors.

In order to study whether the stimulation of the olfactory

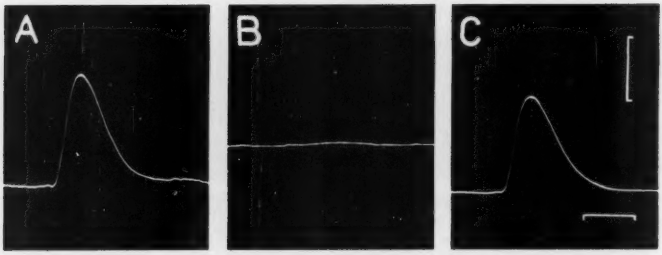

Fig. 35. Records of responses to butanol: A, before; B, after the epithelium had been covered with a thin plastic membrane; C, after removal of the membrane. Stimulus: 0.05 M butanol. Volume of air 1 cc. Vertical line in C 2 mV. Full description in text.

receptors requires contact to be established between the particles of the odorous material and the epithelium, a series of experiments was carried out as follows.

The nasal mucosa was covered with a small piece of a plastic membrane (cf. HAAPANEN and OTTOSON, 1954) which was made so thin that Newton's rings were seen on its surface. If thicker, the membrane was difficult to penetrate with a microelectrode. When applied on the surface of the epithelium the membrane flattened out instantaneously and adhered so smoothly to the mucosa as to be practically invisible. Capillary microelectrodes were used for recording, and the electrode was inserted through the membrane into the mucous layer to a depth of 10—20 μ. The electrode easily passed through the membrane, which apparently did not offer more mechanical resistance than the membrane of a muscle cell. The indifferent electrode was earthed and in contact with the cotton wool, on which the preparation was placed. Butanol (0.1 M) or amyl acetate was used for stimulation.

The results of these experiments are illustrated by the records in Fig. 35. Record A shows the response evoked in the epithelium by stimulation with butanol. The nasal mucosa was then covered with the membrane, through which the microelectrode was inserted into the mucous layer. As illustrated in B, stimulation now failed to evoke a response. If, however, considerable parts of the olfactory epithelium were not covered by the membrane,

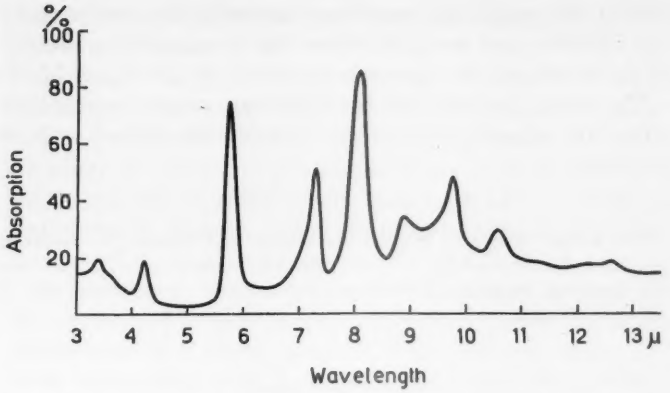

Fig. 36. Diagram showing the infra-red absorption spectrum of the plastic membrane. Ordinate: percentage absorption. Abscissae: wavelength. See text.

a small response was often seen. In order to ensure that the absence of the response was not caused by damage of the receptors, the membrane was removed and the epithelium again stimulated. The response then obtained was usually somewhat smaller than that recorded before the application of the membrane. If, however, the application as well as the removal of the membrane was performed very gently, the difference in amplitude between the two responses was very small, as shown by record C.

Since according to the theory advanced by BECK and MILES (1947) the receptors are stimulated by losses of infra-red radiation, it was of interest to examine to what extent the plastic membrane transmitted waves in this region of the spectrum. The diagram in Fig. 36 shows the percentage absorption curve in the range from 3 to 13.5 μ for a membrane as used in these experiments. As shown by the curve the membrane absorbed the radiation intensely within two narrow bands at approximately 5.7 and 8.1 μ, whereas about 70—80 per cent of the radiation of other wavelengths (in the range from 3 to 13.5 μ) was transmitted. The substances used for stimulation in these experiments (butanol and amyl acetate) have a marked absorption in the region from 8 to 14 μ (see *e.g.* BARNES *et al.*, 1944; RANDALL *et al.*, 1949).

Within this range the membrane transmits the greater part of the radiation and would therefore not prevent the stimulation of the receptors if this occurs as postulated by BECK and MILES.

The results indicate that the epithelium cannot be stimulated unless the odorous particles are brought into contact with the receptors.

The author wishes to express his thanks to Professor N. Svartz for placing at his disposal facilities for infra-red spectroscopy at King Gustaf V's Research Institute. The measurements have been carried out by Mr S. O. Plantin.

H. *Discussion*

It is a common experience that the sensation of odour rapidly weakens and disappears during continuous olfactory stimulation. It has generally been assumed that this adaptation depends on the inability of the olfactory receptors to respond to a prolonged stimulation. However, in studies on the impulse discharge within the olfactory bulb of the rabbit ADRIAN (1950) found no signs of failure of the olfactory receptors at repeated stimulations. On the other hand it was found that there was a competitive interaction between the intrinsic activity of the bulb and the activity induced by the inflow of impulses from the receptors. ADRIAN therefore concluded that olfactory adaptation is due to a suppression of the olfactory signals by the intrinsic activity in the bulb.

The results obtained in the present investigation of the response of the olfactory epithelium indicate that there are reasons to classify the olfactory receptors as slowly adapting end organs. This conclusion is based on the observation that the response to continuous stimulation of low or medium intensity was maintained at a constant potential level throughout the duration of the stimulation. The slow decrement of the height of the response to repetitive stimulation at low or medium stimulus strength further supports this conclusion. As will be thoroughly described in another paper it was found in experiments on the rabbit that the slow potential recorded from the nasal mucosa showed little

signs of adaptation if the olfactory organ was not exhausted by strong stimulation.

Although no direct comparison can be made between the results presented in this paper and those obtained in studies of olfactory adaptation in man, the disappearance of the sensation of odour on continuous stimulation seems more likely to be due to the suppression of the activity induced in the central olfactory pathways (cf. ADRIAN, 1950) than to an inability of the receptors to respond to the stimulation.

It has been assumed that olfactory discrimination depends on the existence of different types of olfactory receptors with selective sensitivities to a limited number of basic odours. However, no such odours have been found. ADRIAN (1948, 1951, 1953) has advanced a theory according to which different odours are recognized owing to a spatial and temporal differentiation of the responses. Olfactory discrimination would thus depend on mechanisms bearing a close resemblance to those associated with visual and auditory discrimination. Since the spatial and temporal distribution of the responses to different substances apparently account for the coarse discrimination only, it has been suggested (ADRIAN 1952, 1954) that the finer differentiation depends on a specific sensitivity of individual receptors to particular molecular configurations.

If there are receptors with such a specific sensitivity, it would be possible to exhaust a fraction of receptors leaving all others less affected. The results obtained in the present study of the interaction of different types of odorous stimuli show that the sensitivity of the olfactory epithelium to particular substances can be selectively reduced. This observation is consistent with the well known phenomenon of selective olfactory fatigue in man, but furthermore indicates that there exist receptors which are specially sensitive to certain substances or to certain molecular characteristics of the stimulating agents. At present it is not possible to draw any conclusions as to the nature of this specificity. Before this can be done the interaction of a great number of substances of different physicochemical properties must be thoroughly analysed. It is probable that a classification of odorous substances may be achieved in this way, but there are many

sources of error involved in this type of experiments and the results have to be treated with great suspicion.

Rhythmic oscillatory potentials are well known phenomena within the central nervous system and are generally regarded as due to a synchronous activity of neurons which are interconnected. It is, however, known that oscillations also occur in the simple eye of *Eledone moschata* (FRÖHLICH, 1914), the retina of which consists of a single layer of visual cells without any nervous interconnections. As already mentioned (see page 27) it has been reported by ADRIAN (1955) that potential oscillations were obtained from the olfactory epithelium in the rabbit. Although the waves observed by ADRIAN have a considerably higher frequency than those seen in the recordings from the olfactory epithelium in the frog it is very likely that they represent identical phenomena. The waves indicate apparently an intermittent synchronous activity in groups of receptors. Since there are no nervous connections between the neurons in the olfactory epithelium the mechanisms of this synchronous activity are not clear. It is possible that the waves arise owing to a waxing and waning state of excitability within groups of receptors of similar functional properties. It has further to be considered that the receptors are densely packed in one layer, and consequently there are favourable conditions for the interaction between active elements. However, there are reasons to assume that the appearance of the waves indicates that the preparation no longer reacts in the normal way to the stimulus. This assumption is based on the observation that usually the waves did not arise until several hours after the dissection or in preparations which were exhausted or damaged.

On theoretical grounds many objections have been raised against the radiation theories of olfactory stimulation (see *e.g.* YOUNG *et al.*, 1948; MONCRIEFF, 1951; GEREBTZOFF, 1953), and attempts have also been made to examine their validity experimentally. In experiments on humans SHKAPENKO and GEREBTZOFF (1951) demonstrated that no sensation of odour could be evoked when the odorous material was separated from the olfactory epithelium by the membrane of a polythene tube inserted into the nasal cavities. Neither do the results obtained

in the present investigation (section G) favour the view that the olfactory receptors are stimulated by losses of infra-red radiation. It has, however, to be taken into account that the theory put forward by BECK and MILES is based on studies on insects. It is possible although not likely that the mechanism of olfactory stimulation in insects differs from that in vertebrates. Certain differences between the structure of the olfactory end organs in insects and that of the receptors in higher animals support such a view. In insects the olfactory receptors are located on the antennae (see *e.g.* DETHIER, 1954). The sensory hairs protrude into a fluid-filled vacuole covered by a membrane which is less than 1 μ thick. This membrane protects the receptors from drying but also seems to prevent the stimulating agent from coming into contact with the sensory elements. There are, however, indications (RICHARDS, 1952; DETHIER, 1954) that the membrane is easily diffusible to gases. If this theory is correct there seems to be little reason to assume that the mechanism of olfactory stimulation in insects differs in some basic respect from that in vertebrates.

General summary

The aim of the present investigation has been to analyse the electrical activity of the olfactory epithelium. The experiments have been carried out on frogs and recordings have been made from the nasal mucosa by means of a direct coupled amplifier. The results are presented as follows:

PART I

A. A slow negative monophasic potential is evoked in the olfactory mucosa when odorized air is blown into the nasal cavities.

B. The response is obtained only from the olfactory area of the mucosa.

C. The response is not abolished by cocaine in a concentration at which the olfactory nerve fibres are paralysed.

D. The response persists during antidromic stimulation of the olfactory nerve fibres.

E. The response led off with a microelectrode inserted into the olfactory epithelium becomes successively smaller the farther from the surface of the mucosa the recording is made.

F. The response is promptly abolished when a small amount of ether or chloroform vapour is blown into the nasal cavities or if water is applied on the mucosa.

G. The response seems to originate in the olfactory receptors. The results indicate that the olfactory hairs are the true sensory elements. In many respects the olfactory sense organ bears a close resemblance to the retina of certain evertebrate eyes.

PART II

A. The amplitude of the response is within certain limits proportional to the logarithm of the stimulus intensity.

B. A similar relationship exists between the amplitude of the response and the volume of the stimulating air at a given stimulus strength.

C. Equal amounts of odorous material distributed in different volumes of air evoke responses of equal amplitudes.

D. The shape and time course of the response is related to the strength of the stimulus. With an increase of the odour intensity of the stimulating air the potential rises at a faster rate, the crest of the response broadens and the decay time lengthens.

E. The "waveform" of the stimulating air current is of great importance for the shape and time course of the response.

F. Substances of different physicochemical properties give responses of different time characteristics.

G. The latency of the response to stimulation with butanol of different stimulus strengths varies from 0.2 to 0.4 sec.

H. The relation between the strength of the stimulus and the amplitude of the response is essentially the same in the olfactory epithelium as in other sense organs.

PART III

A. During continuous stimulation a response is evoked which falls off from the crest to a potential level which is maintained throughout the duration of the stimulation. The height of the constant level in relation to the crest amplitude is lower the higher the stimulus intensity.

B. When the epithelium is stimulated repeatedly at short time intervals the height of the response becomes successively smaller in the course of the first three or four stimulations, the reduction being greater the higher the stimulus strength. The subsequent stimulations at low or medium stimulus strength cause only a slight further diminution of the response.

C. Stimulation leads to a reduced excitability of the epithelium for a period which outlasts the decay time of the response.

D. The sensitivity of the epithelium to different substances can be selectively reduced.

E. Rhythmic oscillatory potentials often appear superimposed upon the top of the slow response evoked at high stimulus strength.

F. The response is often preceded by a small positive deflection, the amplitude of which is related to the humidity of the stimulating air. This deflection is apparently caused by positively charged water particles.

G. No response is obtained on olfactory stimulation if the nasal mucosa is covered with a thin plastic membrane which transmits infra-red radiation but impedes contact to be established between the stimulating particles and the epithelium.

H. The olfactory receptors can apparently be classified as slowly adapting end organs. The selective reduction of the sensitivity of the epithelium indicates that there are receptors with a specific sensitivity to particular substances or molecular configurations. There are no indications that the olfactory receptors can be stimulated unless the odorous material is brought into contact with the epithelium.

Acknowledgement

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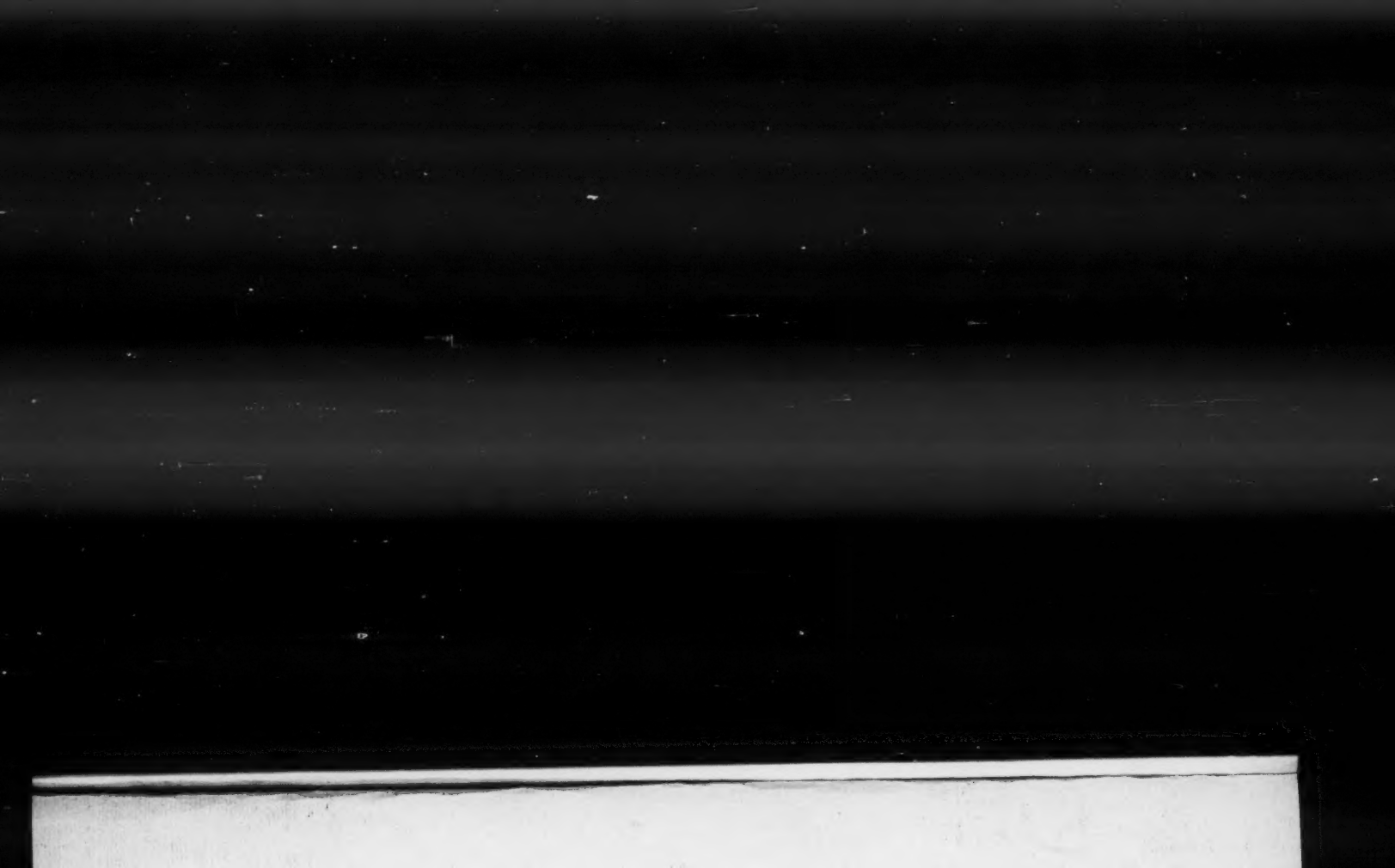
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